

RNA - DEPENDENT DNA POLYMERASE FROM RAT TISSUES:  
DISTRIBUTION, PARTIAL PURIFICATION, AND CHARACTERIZATION

CENTRE FOR NEWFOUNDLAND STUDIES

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RNA-DEPENDENT DNA POLYMERASE FROM RAT TISSUES:  
DISTRIBUTION, PARTIAL PURIFICATION, AND CHARACTERIZATION

A thesis

by



Francesco Moranelli

Submitted in partial fulfillment of  
the requirements for  
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To my Parents

*Ai miei Genitori*

"The inner nature of science within the scientist is both emotional and intellectual. The emotional element must not be overlooked, for without it there is no sound research on however odd and dull-seeming a subject. As is true of all of us, an emotion shapes and forms the scientist's life; at the same time an intellectual discipline molds his thinking, stamping him with a character as a seaman's although much less widely understood." \*

*"La natura interna della scienza in uno scienziato contiene sia l'emozioni che l'intelletto. L'emozioni non si devono sovrastimare, perché senza di esso non c'è nessuna ricerca effettiva su qualunque soggetto, strano o noioso ch'esso possa sembrare. Un'emozione forma e modella la vita di tutti noi e fa la medesima cosa per lo scienziato; allo stesso tempo, la disciplina intellettuale modella i suoi pensieri marcandolo con un carattere da marinaio, però compreso molto di meno." \*\**



\* From "Scientists Are Lonely Men" by Oliver La Farge in "A Treasury of Science", ed. by H. Shapley, S. Rapport, and W. Wright, p. 21, Harper and Row, New York, 1963.

\*\* The author wishes to express his sincerest gratitude to Miss Pompea Maiolo for providing the Italian translation to the above passage.

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## ABSTRACT

RNA-dependent DNA polymerase (RD-DP) has been detected in all rat tissues examined, and a partial purification and characterization carried out. The relative distribution per unit weight of tissue in order of decreasing amount of activity is as follows: thymus, spleen, brain, liver, kidney, testis, heart, plasma, and red blood cells. The activity in crude extracts was found complexed to an endogenous template sensitive to RNase. After partial or complete elimination of the endogenous template, this enzyme was found to respond to externally added templates such as yeast RNA, 16S+23S rRNA from *E. coli* and Q $\beta$ RNA. The rRNA and Q $\beta$ RNA were more efficient templates than yeast RNA. RNase treatment prior to fractionation was found to abolish a portion of the endogenous template without freeing the enzyme from the high molecular weight (MW) complex. Prolonged RNase treatment was found to shift 30-80% of the RD-DP activity to a lower MW region on a gel filtration column, resulting in a separation of the activity from the bulk of the DNA-dependent and endogenous DNA polymerase activities. The characteristics of the low MW RD-DP differ substantially from those of the DNA-dependent DNA polymerase. Mn<sup>++</sup> proved to be twice as efficient as Mg<sup>++</sup> for the RNA-dependent activity with an optimum concentration one-fifth that of Mg<sup>++</sup>, whereas the DNA-dependent polymerase preferred Mg<sup>++</sup> to Mn<sup>++</sup>. A MW of approximately 120,000 has been estimated for the RD-DP eluting in the lower MW region. The activity versus enzyme concentration curve for the RD-DP was sigmoidal, and that for the DNA-dependent DNA polymerase linear, in the concentration range examined. The effect of the rifamycin derivatives on the RD-DP paralleled

their effect on the RD-DP from RNA oncogenic viruses reported in the literature. An interesting observation was the effect of N-ethylmaleimide and p-chloromercuribenzoate; the RD-DP was relatively unaffected compared to the inhibition effect on the DNA-dependent activity. Some of the later experiments revealed a DNA-dependent enzyme eluting in the same region as the low MW RD-DP. As to whether these two activities originate from the same or different enzymes remains to be determined.

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## LIST OF ABBREVIATIONS

ALV	Avian leukosis virus
AMV	Avian myeloblastosis virus
BSA	Bovine serum albumin
pCMB	p-chloromercuribenzoate
cpm	counts per minute
dATP	2'-deoxyribosyladenine-5'-triphosphate
dCTP	2'-deoxyribosylcytidine-5'-triphosphate
DD-DP	DNA-dependent DNA polymerase
dGTP	2'-deoxyribosylguanine-5'-triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acids
DNase	deoxyribonuclease
DOC	deoxycholate
DTT	dithiothreitol
dTTP	2'-deoxyribosylthymidine-5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
FeLV	Feline leukaemia virus
MSV	Murine sarcoma virus
MTV	Mammary tumor virus
MuLV	Rauscher murine leukaemia virus
NEM	N-ethylmaleimide
PHA	phytohaemagglutinin
pI	isoelectric point



pmoles	picamoles ( $10^{-12}$ moles)
RD-DP	RNA-dependent DNA polymerase (reverse transcriptase)
RDD-DP	RNA-DNA-dependent DNA polymerase
RLV	Rauscher leukaemia virus
R-MLV	Rauscher mouse leukaemia virus
RNA (rRNA, yRNA, Q $\beta$ RNA)	ribonucleic acid (ribosomal, yeast, phage Q $\beta$ )
RNase	ribonuclease
RS-DP	RNase-sensitive DNA polymerase (endogenous DNA polymerase)
RSV	Rous sarcoma virus
Sp. Act.	specific activity
Tris	tris(hydroxymethyl)amino-methane

# LITERATURE REVIEW

## A. RNA-DEPENDENT DNA POLYMERASE FROM RNA ONCOGENIC VIRUSES:

### (1) Indirect Evidence for an RNA-dependent DNA Polymerase

The quest for an enzyme capable of synthesizing DNA on an RNA template was perhaps the outcome of an attempt to explain how an RNA tumor virus could possibly produce a stable genetic trait (that is the malignant transformation) that could be transmitted linearly from cell to cell. Due to a number of observations it was suggested (1) that this linear transmission occurred via a DNA provirus, representative of the viral information, that became integrated with the host chromosome. The early evidence, was largely indirect and based on the following observations.

- (i) The antibiotic actinomycin D inhibits the synthesis of RNA made on a DNA template, but not the synthesis of RNA made on an RNA template (1a). However, upon adding the antibiotic to cell cultures infected by RNA viruses, it was found that all RNA synthesis was inhibited, suggesting that the virus might replicate through a DNA intermediate (1, 2).
- (ii) Experiments using stationary cells exposed to Rous Sarcoma virus (RSV) in the presence of inhibitors of DNA synthesis suggested that infection of cells by RSV requires the synthesis of DNA different from that synthesized in the S-phase of the cell cycle (3, 6).

(iii) Cells transformed by RSV contain new DNA which hybridizes with viral RNA, while untransformed cells do not (5).

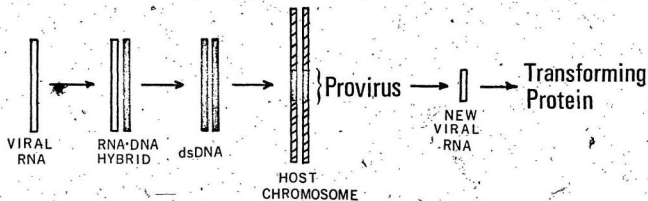
The DNA complementary to the viral RNA came to be known as the provirus and is an essential feature of the provirus hypothesis formulated by Temin (4) (see Scheme I). However, the evidence for this hypothesis was indirect and it was not widely accepted until more direct evidence was made available. In addition, such an information transfer (from RNA to DNA) demanded the existence of an enzyme capable of synthesizing DNA on an RNA template, which was believed by many molecular biologists to violate the *Central Dogma* of molecular biology proposed by Crick in 1958 (7).

(2) Direct Evidence for an RNA-dependent DNA Polymerase

If stationary cells are exposed to RSV in the presence of inhibitors of protein synthesis, the cells still become malignant, suggesting that the enzyme necessary for the synthesis of DNA from an RNA template was already in existence (Mizutani, as communicated by Temin in ref. 8). Consequently, a search was initiated to find such an activity, and in 1970, Temin and Mizutani (9), and Baltimore (10) reported such an enzyme in virions of RSV (9, 10) as well as in Rauscher mouse leukemia virus (R-MLV) (10). These findings were immediately confirmed by others (11, 12) who in addition found the activity to be present in the mammary tumor virus (MTV) of mice (11), and in six different RNA oncogenic viruses (12).

(3) Method of Assay for the RNA-dependent DNA Polymerase

An essential first step in making RNA-dependent DNA polymerase



FORMATION OF PROVIRUS AND OF TRANSFORMING PROTEIN

SCHEME I

dsDNA -- double-stranded DNA

accessible for assay is to disrupt the viral core through the use of low levels of nonionic detergents (12). Such treatments markedly increase the level of activity; however, the acceptable range of detergent concentration is very narrow, since high concentrations ( $>0.2\%$ ) inhibit the activity. Assays have been directed towards measuring both the endogenous templated reaction that was sensitive to RNase A and an exogenous templated reaction utilizing an added RNA template. Both natural and synthetic polyribonucleotides have been used to direct the synthesis of complementary DNA; the synthetic templates, however, are less specific in distinguishing viral and cellular polymerases than are the natural ones. Various criteria need to be met before a DNA polymerase can be declared as RNA-dependent (13, 33): (i) if the activity is endogenous, it must be sensitive to RNase, (ii) the enzyme must require all four deoxyribonucleotides for optimum activity (to eliminate the possibility of terminal addition); (iii) the product must be shown to be DNA, (iv) the product must be shown to be complementary to the RNA template, and (v) the polymerase activity banding at a density characteristic of the virus, should be dependent on detergent treatment.

#### (4) Distribution of Viral RNA-dependent DNA polymerase

An up-to-date list of all the RNA viruses containing RD-DP activity may be found in the review article by Gallo (13). The activity is present in 33 out of 40 RNA viruses, 31 of which are thought to be oncogenic. In addition, the activity was also found in the Visna virus which was previously believed to be non-oncogenic, but recently Takemoto

and Stone (14) have found that this virus could transform cultured mouse cells, hence extending the list of oncogenic viruses. At present the only non-oncogenic virus containing RD-DP activity is the Simian foamy virus. In addition, RD-DP has been found in both "type B" particles from human breast cancer (15) and "type C" particles of human origin, suggesting a relationship between RNA viruses and human cancers (16). Furthermore, Spiegelman et al. (17) have found the activity in a series of human leukaemic cells. Its distribution in all RNA oncogenic viruses examined to date and in some malignant cells, has led to RD-DP being used as a marker for determining oncogenicity.

#### (5) Purification

RNA-dependent DNA polymerase has been extensively purified from avian myeloblastosis virus (AMV) (18, 19), from Rauscher murine leukaemia virus (MuLV) (20, 21), RD-144 cells (20), Schmidt-Ruppin strain of RSV (22), and from human acute leukaemic cells (23). Kacian et al. (18) have purified AMV RD-DP by a combination of column chromatography (DEAE-cellulose, phosphocellulose, hydroxylapatite, and DNA-cellulose chromatography) and gel filtration methods (CM Sephadex), and have shown it to consist of two subunits. The enzyme preparation was free of RNA and DNA endonuclease activities and was active with RNA-, DNA-, and hybrid-templates. The subunit nature of the enzyme has been confirmed by Grandgenett et al. (19) by purifying the AMV RD-DP by phosphocellulose chromatography. Faras et al. (22) have purified the activity 500-fold from RSV and obtained two active fractions by phosphocellulose chromatography. These investigators subsequently reduced RNase contamination a great deal by gel filtration chromatography on Sephadex G 100 (22). Other workers have purified the activity by affinity chromatography on

dt<sub>12-18</sub>-cellulose columns (20), and on a solid phase immunoadsorbent consisting of antibody, directed against MuLV, bound to Sepharose 4B (21). The immunoadsorbent column was successful in purifying all RD-DP activities antigenically related to the MuLV RD-DP activity. The dt<sub>12-18</sub>-cellulose column on the other hand was very successful in separating viral DNA polymerases from cellular DNA polymerases.

#### (6) Characterization

After the RD-DP activity was detected in RNA oncogenic viruses, a number of workers directed their research towards characterizing this activity. As expected for a DNA polymerase, the enzyme required the presence of all four deoxyribonucleoside triphosphates for maximum activity (9, 10, 12, 24, 25). The enzyme did not polymerize ribonucleotides (9, 10, 12, 25).

With regard to divalent cation requirements, both  $Mg^{++}$  and  $Mn^{++}$  may serve as activators although to different extents (9-12, 18, 22, 24, 25) whereas  $Ca^{++}$  could not (9). The relative efficiency of the two cations has varied depending on the viral system from which the RD-DP was isolated, as well as its extent of purity. Leis and Hurwitz (24) have shown that in the case of AMV RD-DP, the optimum  $Mg^{++}$  concentration was 10 mM, and could be only partially replaced by  $Mn^{++}$ . When enzyme preparations were contaminated with RNase, however, the activity with  $Mn^{++}$  was greater than with  $Mg^{++}$ . Furthermore, the presence of both cations displayed a synergistic effect. In the case of RSV RD-DP (9, 22), it has been observed that both cations stimulate identically, and the presence of both had no synergistic effect on the activity.

RSV RD-DP requires the presence of dithiothreitol (DTT) or mercaptoethanol for maximum activity (9, 10, 18, 24) and is highly sensitive to sulfhydryl reagents (26). The presence of bovine serum albumin (BSA) (200 µmg/ml) was found to stimulate the RSV RD-DP two-fold (22). Monovalent cations enhance the activity from AMV (12, 18, 25), whereas in the case of the activity from RSV (9, 22) contradictory observations have been made. Temin and Mizutani (9) observed a 15% stimulation by 20 mM KCl and an inhibitory effect at higher concentrations, whereas Faras et al. (22) found that monovalent cations inhibited the activity at all concentrations. These contradictory results may be due to differences in ionic strength of their reaction conditions.

The activity from AMV had a broad pH optimum (pH 7.8-9) with a maximum at pH 8.2 (24), whereas the optimum for RSV lay between pH 8-9.5 (9). The temperature optimum for RSV RD-DP lay between 40-50°C (9).

Duesberg et al. (27) have estimated an isoelectric point below 6 for the RD-DP from RSV, on the basis of the ionic strength required to elute the enzyme from a DEAE-cellulose column. Molecular weights have been estimated for enzymes from the various sources, and have varied somewhat. RD-DP from human leukaemic cells has a MW of 130,000 (23), that from RSV 110,000 (27); that from AMV 110,000 (18) and 160,000 (24, 26), and that from MuLV 70,000 (28), and 90,000 (24, 26).

Below a critical concentration, the enzyme was found to lose all perceptible activity (22, 24) suggesting that the enzyme may be inactivated by dissociating into constituent subunits. This has been shown to be the case (18, 22).



With regard to kinetics, Spiegelman et al. (12, 25) have observed that the RD-DP activities from RSV, and Rouscher leukaemia virus (RLV) maintain linear synthesis for time periods extending up to 8 hours, whereas that from AMV, Feline leukaemia virus (FeLV), and MTV tend to slow down after approximately 90 minutes.

Perhaps one of the most interesting properties of the AMV RD-DP is its relatively high frequency of errors when copying poly(rA).poly(dT) (30). DNA polymerases from human leukaemic lymphocytes also appeared to be more mutagenic than the activity from normal ones (29). These observations have led the authors to suggest that mutagenicity may be a key factor for tumor progression since it could lead to the eventual production of faulty enzymes, including DNA polymerases.

#### (7) Template Specificity

A number of workers (28, 31-36) have examined the ability of viral DNA polymerase to utilize a series of both natural and synthetic templates for the synthesis of DNA. These studies had the following objectives: (i) to attempt to detect a DD-DP which would explain the double-stranded nature of the final product (31, 32), (ii) to establish a difference between template specificity of cellular and viral DNA polymerases (33, 35), and (iii) to make available a highly sensitive assay for the viral DNA polymerase which would at the same time distinguish between viral and the cellular enzymes (28, 33-35).

Spiegelman et al. (36) have examined the efficiency with which single- and double-stranded RNA as well as double-stranded DNA and DNA-RNA hybrids were used as templates by the RD-DP from AMV. Of the double-stranded

synthetic templates, the ribohomopolymers were superior to the corresponding deoxypolymers, whereas of the DNA-RNA hybrids poly dC.rG and poly dI.rC were the best templates. In general, single-stranded homopolymers were found to be poor templates unless a complementary oligomer was added, and double-stranded homopolymers were generally better templates than the corresponding single-stranded ones. In addition, DNA from chicken embryos was found to be a much better template than the endogenous RNA. Similarly Mizutani et al. (32) have observed that native DNA from both calf-thymus and *E. coli* was used as a template for DNA synthesis by viral DNA polymerase. However, the activity was reduced two-fold upon denaturation of the DNA template.

With regard to natural RNA templates, Mizutani et al. (32) have found that AMV RD-DP could mediate synthesis of DNA complementary to QBRNA, Maloney sarcoma viral RNA, and AMV RNA. Similarly, Duesberg et al. (28) have found that RSV RD-DP used polyribonucleotides from RSV, influenza virus, tobacco mosaic virus, and ribosomes. In addition they found that if the 60-70S RSV RNA was heat dissociated, there was a 5-10-fold decrease in its efficiency as a template. However, if oligo (dT) or oligo (dC) was added to the heat dissociated RNA, the activity was enhanced 20-30-fold. A 15-fold stimulation has also been observed upon addition of oligo (dT)<sub>10</sub> to the AMV 70S RNA (35). These results suggest that a primer is required for template activity.

Goodman and Spiegelman (33) and Robert et al. (35) have compared the response to various templates by both viral and cellular DNA polymerases. It was found that the viral enzyme in addition to responding to

single-stranded heteropolyribonucleotides, greatly preferred poly(A). (dT)<sub>10</sub> as a template whereas the *E. coli* polymerase used poly(dA).(dT)<sub>10</sub> with an efficiency equal to or greater than the efficiency with poly(A).(dT)<sub>10</sub>, but showed no activity with the single-stranded polyribonucleotides Q $\beta$ RNA, and 70S viral RNA. Robert et al. (35) compared the activities from AMV and Mason-Pfizer monkey virus to those of normal human lymphocytes and *E. coli* DNA polymerase I. Both viral and cellular enzymes responded equally well with the 'non-specific' double-stranded template poly(rA).poly(dT). The viral enzyme showed a preference for poly(rA).oligo(dT)<sub>12-18</sub> while the cellular enzymes preferred poly(dA).oligo(dT)<sub>12-18</sub>. A distinction between viral and cellular enzymes can thus be made on the basis of template specificities if a number of synthetic and natural templates are used.

#### (8) Other Activities Associated with RD-DP

A necessary prerequisite for the integration of the DNA complementary to the viral RNA into the host cell genome is that the DNA must be of a double-stranded nature. This, however, requires the presence of a DNA-dependent DNA polymerase (DD-DP) as pointed out by Spiegelman (25). Hence various workers (31, 32, 37, 38) have examined a series of RNA oncogenic viruses and have indeed found such an activity. In addition they have shown it to prefer double- to single-stranded DNA (37). The activity from AMV prefers a DNA template rich in G and C residues (31). In addition to a DD-DP activity, an endonuclease activity has been found (32) and these same workers have also speculated on the existence of a ligase. Smoler

et al (69), however, have failed to detect the ligase activity. In addition various other workers (19, 39-43) have found a RNase H<sup>+</sup> (Hybridase) activity in a number of viruses similar to the one found by Hausen and Stein (44) in calf-thymus. Grandgenett et al. (19) have attributed this activity to a single subunit possessing RNA-dependent activity as well. Both the RNA-dependent and DNA-dependent activities are believed to originate from the same catalytic site (28). This is based on the observation that when partially purified RLV polymerase is saturated with RNA template, there is no increase in the activity when DNA is added (unpublished results of Reitz, M., Sarin, P., and Gallo, R., communicated by Gallo in ref. 13).

#### (9) Inhibition Studies

(a) Antibiotics. The effect of inhibitors on the viral RD-DP has been studied by a large number of workers. Such studies were carried out for a variety of reasons: (i) specific inhibitors of RD-DP might be used to determine the biological function of RD-DP (see below); (ii) they may provide an important group of drugs for the chemotherapy of viral diseases and cancer, and (iii) they may provide information concerning the nature of the active site of the enzyme.

One of the most extensively studied group of antibiotics are the rifamycin derivatives (45-48). Gurgo et al. (45) have examined the effect of 180 rifamycin derivatives on the RD-DP from MSV, and have noted that lengthening of the 3-side chain of rifamycin by either aromatic or aliphatic substituents rendered the inhibitors more efficient. In addition they have shown that the *ansa* ring (the naphthoquinone ring) (for structure see Appendix I) is required for inhibition, since the polymerase is not

inhibited by the free aminopiperazines corresponding to side chains of active derivatives. These data suggest that the *ansa* ring structure recognizes a structural feature of the RD-DP. Smith et al. (47) and Yang et al. (48) have also examined the effect of a spectrum of such derivatives on viral RD-DP, and have classified them according to their potency. Such antibiotics have been used to distinguish between cellular and viral DNA polymerases (47, 48) and to determine the biological role of RD-DP (46). Another class of antibiotics, the streptogramins, have also been used to determine the enzyme's biological function (49). Other inhibitors which possess a significant inhibition capacity towards viral RD-DP are the anthracycline derivatives (50), and ethidium bromide (51, 52). On a molar basis, ethidium bromide was a more effective inhibitor of RD-DP than the most active rifamycin derivatives (52); it lacks specificity, however, and acts by binding to nucleic acids, and not to the enzyme (51, 53, 54).

(b) Antibodies. Antibodies against viral RD-DP have been used to examine the relationship among RD-DPs from various RNA oncogenic viruses as well as their relationship to cellular DNA polymerases (55, 56). Aaronson et al. (55) have prepared an antibody against murine RD-DP and have shown it to cross react with polymerases from some mammalian "type C" RNA viruses (i.e. feline, rat, and hamster), but not with polymerases from avian "type C" or any "type B" RNA viruses. The antibody did not cross react with cellular DNA polymerases. Todaro and Gallo (56) have shown that an RD-DP from human acute myeloblastic leukaemic cells was immunologically related to the RD-DP from known "type C" leukaemia viruses.

and in particular to the enzyme from primate "type C" virus. Gerwin et al. (57) have made use of an antibody against RD-DP from MSV to localize the RD-DP in the nucleoid.

#### (10) Nature of Product

The product of the RD-DP reaction was clearly shown to be DNA by various criteria: susceptibility to DNase, resistance to RNase and alkaline hydrolysis (9, 10, 12, 24, 25, 37) and solubilization by heating in 1 N HCl (24). Nearest neighbour frequency analysis has revealed it to be heteropolymeric (12, 58), and annealing experiments complementary to the RNA template (12, 23, 25, 32, 34, 38). In  $\text{Cs}_2\text{SO}_4$  density gradients, the product of a long reaction time migrated to a density somewhat greater (1.450) than the density of DNA from mouse embryo fibroblasts (1.420) (12, 25). In general, early reaction times have yielded a product that banded in the RNA density region indicating it to be complexed to the template, whereas longer reaction times generally gave a series of hybrid structures banding between the RNA and DNA density regions (12). Denaturation of the product has yielded various results depending on the methods used: If heat was used, some of the product remained attached to the RNA; however, if alkali was used the product was found entirely in the region of DNA on a  $\text{Cs}_2\text{SO}_4$  density gradient (23, 59, 60). Heat plus RNase treatment also released the product to the appropriate region (59). These results suggest a covalent link as well as hydrogen bonding between the DNA product and the RNA template.

Some workers also studied the nature of the secondary structure of the product. Generally, two approaches were taken: the first consisted of

fractionating the product on hydroxyapatite after digesting away all RNA, and the second was treatment of the product with a single strand-specific nuclease (exonuclease I) of *Neurospora crassa*. In the first approach a large portion of the product was retained on the column, indicating a double-stranded DNA (38, 58). The second approach confirmed this since the nuclease had no effect on the product unless it was first denatured (38, 58). The size of the product was also estimated (23, 24, 25, 59) and it was found to be very small relative to the viral RNA template used. Indeed, Leis and Hurwitz (24) have shown it to represent only 3% of the added RNA. However, under different conditions Duesberg et al. (61, 68) have found that between 70 and 80% of the product was homologous to the RNA template when an excess of DNA product was used for hybridization, indicating that most of the RNA genome is represented in the product. Varmus et al. (63) arrived at a similar conclusion by studying the re-association kinetics of the product which indicated that the product represented approximately 70% of the template. The double stranded nature of the product has made hybridization experiments difficult, hence Ruprecht et al. (62) have devised a method in which only DNA complementary to RNA would be synthesized. This consists essentially of carrying out reactions in the presence of high concentrations of actinomycin D in the case of G-rich templates, and in the presence of distamycin A in the case of T-rich templates. These inhibitors prevent the synthesis of double-stranded DNA hence assuring synthesis of only DNA complementary to RNA.

(11) Biological Function of RNA-dependent DNA-Polymerase

The very fact that the enzyme was present in the RNA oncogenic viruses, and its ability to transfer information from RNA to DNA, suggested that it might play a role in the initiation of neoplastic transformation. Hanafusa and Hanafusa (64) have provided direct evidence that this is the case by isolating a RSV mutant [designated RSV<sub>α</sub> (0)] that was noninfectious and could not transform susceptible chick cells. Upon examining this mutant for RD-DP activity they found that it was absent, hence providing direct evidence for its biological function. Furthermore their results suggested that the enzyme in RSV particles is not a host cell enzyme which is incorporated into virions as they mature. The simultaneous addition of RSV<sub>α</sub> (0) with avian leukosis virus (ALV) which replicates in but does not transform the chick cells, resulted in transformed fibroblasts. The results were interpreted as meaning that the inability of RSV<sub>α</sub> (0) to replicate and transform chick cells is due to a mutation in the gene(s) specifying RD-DP. In the presence of ALV, the RSV<sub>α</sub> (0) was able to use the ALV RD-DP and consequently able to transform the cells. Noninfectious viruses were also produced by the transformed cells suggesting that the polymerase is required for transformation but not for the maintenance of the transformed state.

Another approach taken in establishing the biological function of RD-DP was the use of specific inhibitors. Carter et al. (49) have made use of the RD-DP inhibitor, streptovaricin to block transformation of mouse fibroblasts by MSV. In addition other workers (65, 66; 67) have established a direct correlation between inhibition of RD-DP activity through the use of



rifamycin derivatives and the loss of the capacity to transform rat cells.

A scheme of the possible ways in which RD-DP from RNA viruses may carry out its biological function is depicted in Scheme II. The scheme accounts for all the activities believed to be present in such viruses and represents them as being distinct, although some of the activities are believed to originate from the same enzyme. In fact, some of the reactions may be concerted. The scheme is based on the observation of the various activities as well as on the nature of the products formed (see part 8 and 10 of this section).

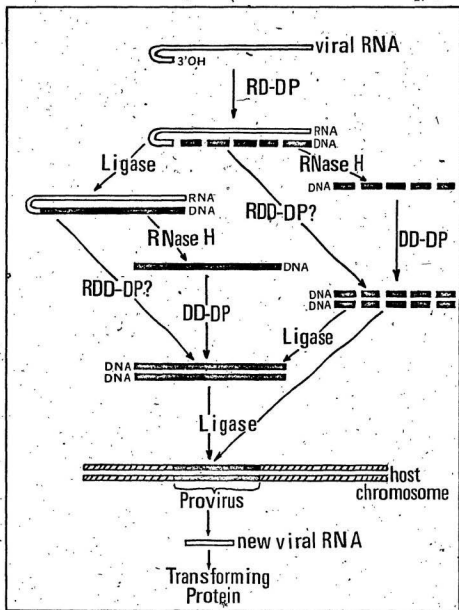
#### B. RNA-DEPENDENT DNA POLYMERASE FROM NON-MALIGNANT CELLS:

##### (1) Introduction

The finding of an RD-DP in RNA oncogenic viruses and its apparent presence in normal cells made it tempting for a number of workers (see for example 70, 71) to speculate on the possible functions of such an activity in normal cells. The nature of the reaction catalyzed made this activity a likely candidate for the possible amplification of genes (86-90), cell differentiation (77, 94-97), antibody formation (126), and memory (70, 71). Consequently a number of workers have attempted to detect such an activity in various biological systems. In addition, the potential use of such an enzyme as a marker for oncogenicity made it necessary to examine whether its presence was limited to RNA oncogenic viruses, or whether it was more widely distributed (72).

##### (2) Assay Methods Used in Detecting RD-DP

The general approach in detecting RD-DP activity in normal cells is



POSSIBLE MODES OF VIRAL INFORMATION TRANSFER TO THE HOST CHROMOSOME,  
AND SUBSEQUENT TRANSFORMATION OF THE HOST CELL.

essentially the same as that used in assessing viral RD-DP activity. Like the assay for the viral enzyme, RD-DP activity from some eucaryotic systems has been confirmed through its sensitivity to RNase (77, 79, 80), its response to naturally occurring polyribonucleotides (72-76, 81-83) and mainly to synthetic ribohomopolymers (92-104). Unlike the viral enzyme, treatment with nonionic detergents is not essential to make the activity accessible, and a number of the reported activities have not been found to respond to natural RNAs although they do respond to synthetic ribohomopolymers (92-104). This phenomenon raises the possibility of two different classes of RD-DP activities in eucaryotic cells, one capable of responding to heteroribopolymers and the other to homoribopolymers. It is possible however that the lack of response to natural RNAs may be an *in vitro* phenomenon, and that *in vivo* the enzyme does in fact use heteropolyribonucleotides.

It is now apparent that labels such as "RNA-dependent" and "DNA-dependent" are not really appropriate for describing specific enzymes because pure DNA-polymerases have been shown to use both RNA and DNA templates (81-83), although with different efficiencies. These terms therefore describe an enzyme function, rather than unique enzymes.

The problems associated with synthetic templates, such as the lack of specificity of enzymes towards such templates, is greater than that due to nonspecificity towards natural RNAs. For this reason the RNase-sensitive and heteropolyribonucleotide-dependent activities will be considered separately from those dependent on synthetic templates.

### (3) RNase-sensitive and Heteropolyribonucleotide-dependent Activities

(a) Distribution. DNA polymerase activities, dependent on externally added natural RNA templates, have been found in the following biological systems: PHA-stimulated normal human lymphocytes using yRNA as the template (72, 73, 75, 85), chick embryo brain using purified RNA from chick embryonic skin (74), mitochondria from rat liver and cerebral cortex using rat liver RNA (75), and in *E. coli* using 5S RNA and rRNA from rat liver (81), *E. coli* rRNA (82), 28S rRNA from *Drosophila* and tobacco mosaic virus RNA (83). In addition, an activity from chick embryo has been discovered which uses globin mRNA and 60-70S AMV RNA; however, only the poly rA regions of these templates were transcribed (76).

The endogenous (or RNase-sensitive) activity has been found in chicken embryos (77), uninfected rat cells (79), and in PHA-stimulated normal human lymphocytes (80). Purified enzymes from these systems, however, were incapable of using externally added natural RNA templates, although they were capable of using homoribopolymers as templates.

(b) Purification. Initial attempts at detecting RD-DP activity in crude extracts of PHA-stimulated normal human lymphocytes were unsuccessful (66, 72, 85); however, upon partial purification, for example by isoelectric focusing, the activity became apparent (72, 85). In addition it was resolved from the DD-DP activity (72). In contrast to this, the activity in a mitochondrial preparation from rat liver and cerebral cortex (75) was detected without further purification. Very little additional work with regard to purification has been reported, although Soriano (74) has partially purified RD-DP from chick embryo brain (mitochondria and nuclei) by ammonium sulfate fractionation.

One line of evidence, the finding of RD-DP activity in *E. coli* DNA polymerase-I preparations (81-83) purified essentially to homogeneity (83), suggests that the RD-DP activity may be due to the same enzyme that uses DNA as a template. Whether this will also turn out to be the case in eucaryotic cells is not known.

The RNase-sensitive DNA polymerase (RS-DP, or endogenous activity) from chick embryos (77) and normal human lymphocytes (80) have been purified by either density-gradient (80) or a combination of density- and velocity-gradient centrifugation (77). In both studies this activity appeared to be present in a particulate fraction, in contrast to the DD-DP, the bulk of which remained in the soluble fraction.

(c) Characterization. All RD-DP activities using natural RNAs as templates, whether endogenous or exogenous, have been shown to require all four deoxyribonucleoside triphosphates for maximum activity (75, 77, 80, 81, 83). The only exception to this was the RD-DP activity reported by Rougeon et al. (76) in chick embryo. This activity only copied the poly (rA) region of globin mRNA and 60-70S AMV RNA.

With regard to divalent metal requirements, the reported activities were activated by the presence of either  $Mg^{++}$  or  $Mn^{++}$  (77, 79-81), or were completely dependent on  $Mg^{++}$  (75,83). The *E. coli* activity had a  $Mg^{++}$  optimum at a concentration of 6 mM (83), and the endogenous activity from chicken embryo at 15 mM (77).

The extent of synthesis for the *E. coli* RD-DP activity was proportional to the amount of RNA template in the assay mixture, and was linear for approximately one hour, and plateaued by two hours (83).

With regard to the endogenous activities, evidence that the template RNA is as follows: The activity was sensitive to RNase (77-80), resistant to DNase (77), and the chicken embryo endogenous activity (77), and that from stimulated normal human lymphocytes (80) was partially resistant to actinomycin D. The properties of these RNA-dependent polymerases differ from those of viral enzymes. For example, the chicken embryo enzyme (77) was shown to be resistant to an antibody directed against AMV RD-DP. The RD-DP from uninfected rat cells was not activated by Nonidet (99) as viral enzymes are, and N-demethylrifampicin had no effect on the activity from stimulated normal human lymphocytes (80).

(d) Template Specificity. The large variety of DNA polymerase activities discovered in mammalian systems, as well as the discovery of RD-DP in RNA oncogenic viruses, has resulted in a nomenclature for DNA polymerases based on the nature of the template used. Consequently, the response of DNA polymerases to various templates has been described in a number of recent reports.

Many of the enzymes that use natural RNAs as templates (72-75, 81-83) also accept synthetic polyribonucleotides as templates. Both single-stranded homopolymers in the presence of complementary oligomers, and double-stranded polynucleotides have displayed activity. The addition of oligomers to natural RNA templates has had different effects depending on the template (and enzymes) involved. For example, the addition of oligo (dT)<sub>6-9</sub> to 28S RNA from *Drosophila* did not enhance the activity using *E. coli* RD-DP, whereas its addition to tobacco mosaic virus RNA enhanced its activity (83).

All of the endogenous activities reported (77, 79, 80) have been shown to be sensitive to RNase, suggesting that the template was RNA. The possibility that RNA served as a primer for the DD-DP was overcome by hybridization experiments (77, 79). After purification, however, none of these enzymes were capable of using externally added polynucleotides (i.e. natural RNA templates) other than DNA. Like the exogenous activities these enzymes were also capable of using synthetic templates.

(e) Nature of Product. The product of the RD-DP activities from eucaryotic and bacterial cells, like that of the viral enzyme, was shown to be DNA on the basis of acid insolubility, resistance to RNase and alkali, and susceptibility to DNase (75, 77, 81, 83). The initial product behaved like a DNA-RNA hybrid (81, 83) while longer reaction times resulted in a DNA-like product completely susceptible to DNase (81). Bobrow et al. (80), however, could not detect the RNA-DNA hybrid intermediate with the activity from PHA-stimulated normal human lymphocytes. Their failure to do so was probably due to an activity in the enzyme preparation (whose presence they confirmed) identical to RNase H, which degrades such hybrids, hence explaining the absence of the hybrid. The product of chick embryo RD-DP is a mixture of single- and double-stranded DNA (77-79). Kang and Temin (77, 78) have also analyzed in detail the product of a reaction that was carried out in the presence of actinomycin D. Sucrose density gradient analysis revealed sedimentation coefficients of 30S and 6S. The fast sedimenting product, however, disappeared when treated with RNase, alkali, or heat. In addition, that part of the product (35%) which

banded in the RNA region, on a CsCl gradient, disappeared upon treatment with alkali, and subsequently banded in the density range between 1.40 and 1.55 g/cm<sup>3</sup> (i.e. the density range for DNA). When it was heated for 10 minutes at 100°C, much of the peak in the RNA region disappeared; however, some remained and if this was subsequently treated with RNase A and T<sub>1</sub> it disappeared. This suggested both hydrogen- and covalent-bonding between the template and the product (78). When the product was treated with S<sub>1</sub> nuclease the density profile had two peaks, one in the RNA region, and one in the DNA region, suggesting that the product contained both DNA-RNA hybrids, as well as double-stranded DNA. The density of the RNA-DNA hybrid after treatment with S<sub>1</sub> nuclease may be the result of double-stranded RNA remaining attached to the hybrid (78).

With regard to the size of the product using *E. coli* polymerase I, Loeb et al. (83) have observed that it sediments in the regions of 3-6S and 12S, in a sucrose gradient. In addition these workers have shown that 85% of it hybridized to the template. Kang and Temin (77) have found that the product of the endogenous chicken embryo activity hybridized 40% with the RNA from the chicken cell fraction, but did not hybridize to RNA from ALV and RSV.

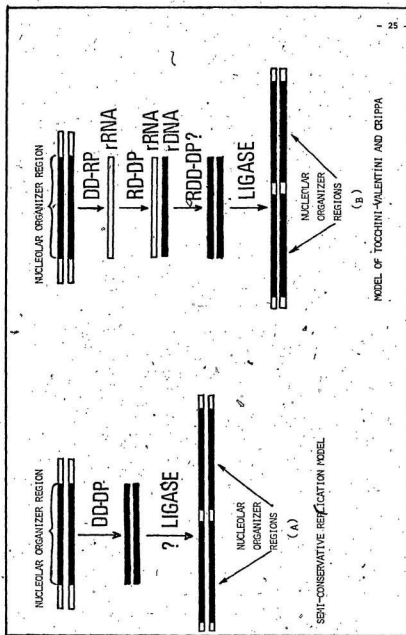
(f) Role in Gene Amplification. The process of ribosomal gene amplification may occur through two possible distinct routes. The first of these and the simplest, postulates that the entire ribosomal DNA complement of the chromosomal nucleolar organizer is used as template for DNA replication, in the classical semi-conservative manner (see Scheme III A). An alternate route has been proposed by Tocchini-Valentini and



Crippa (86, 87) who suggest that the first step in the amplification process is the formation of an RNA transcript of the entire ribosomal DNA unit which then serves as a template for the RD-DP, yielding an RNA-DNA intermediate which would subsequently be converted to a double-stranded DNA. Finally, polycistronic ribosomal DNA would be linked together by a ligase (see Scheme III B). Their hypothesis is based on a series of observations made with *Xenopus* oocytes at the pachytene stage (when gene amplification occurs).

- (i) When DNA from *Xenopus* oocytes at the pachytene stage (labelled with  $^{14}\text{C}$ -thymidine) is extracted and analyzed in CsCl gradients two distinct bands are observed. The heavy band corresponds to ribosomal DNA [because of its high G-C content (123)] and the lighter one to chromosomal DNA. When the oocytes are incubated in the presence of both  $^3\text{H}$ -uridine and  $^{14}\text{C}$ -thymidine, a peak of  $^3\text{H}$ -uridine incorporation bands in the region of ribosomal DNA. This material is resistant to RNase unless first denatured, suggesting that the product is an RNA-DNA hybrid (86-88).
- (ii) Additional evidence came from the observation that the rifampicin derivative 2'5'-dimethyl-N(4')benzyl-N[desmethyl]rifampicin, ( $\text{Me}_2\text{BzRif}$ ) which is known to inhibit RD-DP (127) inhibited formation of the heavier peak but not the lighter one (86, 87). This was confirmed autoradiographically by Ficq and Brachet (89) who have shown that the amplification of rDNA which takes place in the extrachromosomal "caps", was inhibited by the same drug.
- (iii) The isolation of an RNA-DNA hybrid complex (88, 90) as well as the discovery of an enzyme capable of using the rRNA from the complex, as a template for DNA synthesis (88), further substantiated

SCHEME 111.  
MODES OF RIBOSOMAL GENE AMPLIFICATION



this hypothesis.

Although the series of papers that have just been mentioned in relation to gene amplification provide substantial evidence that RD-DP may be involved in gene amplification, Bird et al. (91) have been unable to confirm these results. They have not been able to detect either the RNA (47S) template, or the hybrid intermediate. In addition these workers have shown that the radioactivity after uridine labelling is attributable to the conversion of uridine to deoxycytidine and subsequent incorporation into DNA. This explanation is not satisfactory, however, since it had been shown that 95% of the uridine counts were sensitive to alkali (90) and furthermore were sensitive to RNase A after heat denaturation (88, 90) suggesting that in the system of these workers uridine was not converted to deoxycytidine. It is conceivable that these contradictory observations are a result of the two groups looking at different stages of the amplification process.

#### (4) Synthetic RNA-dependent DNA polymerases

Essentially all biological systems which have been examined contain DNA polymerases capable of using synthetic RNA or RNA-DNA hybrid templates (92-104). It is not known whether these synthetic RD-DPs represent a new class of DNA polymerases or merely an activity of known DD-DPs. Both are probably correct since some of the described activities use DNA as a template while others do not. Certainly the separation of poly RNA-dependent DNA polymerase from the DD-DP activity in chick embryo (94, 95) WI cells (human embryonic lung tissue culture, 98) and rat tissues (100) is consistent with this being a new class of enzymes.

The activity from chick embryo (94) failed to use the natural RNA templates: *E. coli* 16S RNA, rat liver RNA, Q $\beta$ RNA, and chick embryo RNA. A similar failure to use natural RNAs was reported by Bolden et al. (102) for poly rA-dT dependent polymerases in several eucaryotic systems. Rougeon et al. (76) have found that the activity from chick embryo transcribed only the poly rA region of globin mRNA and 60-70S AMV RNA. Fry and Weissbach (99) have shown that even the addition of the oligomer (dT)<sub>12</sub> to a series of natural templates did not allow the enzyme from cultured murine cells to use these RNAs as templates.

Bolden et al. (102) have pointed out that the enzyme preparation from Hela cells may contain an inhibitor preventing it from using natural RNA templates, since the preparation strongly inhibited the ability of AMV RD-DP to copy Q $\beta$ RNA. Furthermore, they have shown that this inhibitor is not a nuclease for if Q $\beta$ RNA is preincubated with the preparation and the inhibitor in the preparation is subsequently destroyed by heating at 60°C, the Q $\beta$ RNA is still used as a template by AMV RD-DP.

Different groups of workers have studied various other characteristics of the synthetic RD-DPs. Evaluation of this information is hampered by the fact that different enzymes capable of using synthetic templates were probably described. In general, however, it appears that Mn<sup>++</sup> is a more effective metal cofactor than Mg<sup>++</sup> (92, 98, 100, 103), dithiothreitol is required to stabilize the enzyme (92, 98) and the MW is probably quite low [50,000 (94) and 27,000 (96) have been reported for the enzyme from chick embryo].

PURPOSE OF, AND APPROACH TO THIS STUDY

Since the finding of an RNA-dependent DNA polymerase in oncogenic RNA viruses, various workers (72-83, 85, 88, 90, 92-104) have described such an activity in animal cells. The possible functions that such an enzyme may be involved in (gene amplification, differentiation, memory and antibody formation), needless to say, has stimulated this present work in which the characteristics and distribution of this enzyme activity have been studied.

At the time this study was undertaken there only appeared several reports in the literature (72, 92) that suggested its presence in untransformed mammalian cells. One of these reports (92), as has been pointed out earlier, was based on the observation that poly rA:dT was capable of acting as a template for DNA synthesis. This evidence however, encompassed several weaknesses, the first being the non-specificity of synthetic templates (i.e., DD-DP might also be capable of using such synthetic templates), so that in effect a novel enzyme might not be involved at all. Secondly, the nature of the template itself (poly rA:dT) presented a situation quite different from what might actually occur *in vivo*; and thirdly, the work was carried out with cultured cells which may not be similar to normal or untransformed cells, although they do resemble cancerous cells in certain aspects (i.e., rapid growth). Furthermore, due to the wide distribution of oncogenic RNA viruses such as type C viruses, the use of cell cultures involves the risk of infection by such viruses, and consequently casts some doubt as to the source of the enzyme. Hence, such systems must be looked upon critically and for this reason freshly prepared tissue homogenates have an advantage.

On the other hand, the work of Penner et al. (72), although having the same weakness associated with the use of cell cultures, did have the advantage of making use of yRNA as a template for DNA synthesis, although it has been suggested (13) that this RNA may have been contaminated with DNA. In any case their work did demonstrate a separation of the RNA-dependent from the DNA-dependent polymerase activities, and suggested the type of system that would most likely prove successful in carrying out initial experiments.

The system used by Penner et al. (72) consisted of an actively proliferating human lymphocyte culture that had been stimulated with PHA. It became apparent from the observations made by these workers that an actively proliferating *in vivo* system consisting of lymphocytes or similar immunocompetent cells might be most suitable. Such a system was provided by the rat thymus, which in addition to other cell types, consists of lymphocytes, or thymocytes as they are known in this organ, and which is actively engaged in lymphopoiesis (109). Therefore this project was initiated with the thymus as a possible source of the enzyme and, as templates, yRNA and later rRNA from *E. coli* were used in order to overcome the problem associated with synthetic templates.

This system having proved successful, various studies, such as the efficiency with which various polyribonucleotides from different sources are copied by the enzyme, as well as the characteristics of the enzyme itself, were undertaken and, in addition, some of its properties were compared to those of the DD-DP. Furthermore, the RD-DP activity has been partially purified from the DD-DP, and its presence extended to all

rat tissues examined. In addition, the current interest in the use of specific inhibitors of viral RD-DP for cancer chemotherapy has led to an investigation of their effect on the mammalian RD-DP.

#### NOMENCLATURE

The term *endogenous activity* has been used to refer to activity sensitive to RNase A, observed in the absence of an externally added RNA template. The term has been used interchangeably with the term *RNase-sensitive DNA polymerase (RS-DP)*. The term *RNA-dependent DNA polymerase (RD-DP)* designates activity observed in the presence of an externally added RNA template, such as yRNA or 16S+23S rRNA. The *low MW RD-DP* is the activity eluting in the 120,000 MW region of a Sephadex G200 column, and the *high MW RD-DP* is the activity eluting in the void volume. *DNA-dependent DNA polymerase (DD-DP)* refers to activity observed in the presence of "activated" DNA.

# MATERIALS AND METHODS

## A. MATERIALS

### (1) ANIMALS, BIOCHEMICALS, AND ENZYMES

- a. ANIMALS: Female rats (100 - 150 gm) of the Sprague-Dawley strain were used for all experiments, and were obtained from either the Animal Unit, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, or from Canadian Breeding Farm and Laboratories Ltd., St. Constant, Quebec.
- b. DEOXYNUCLEOSIDE TRIPHOSPHATES (dATP, dCTP, dGTP, and dTTP) were obtained as their sodium salts, from Sigma Chemical Co., St. Louis, Mo. Stock solutions of 50 mM were prepared in distilled water, and stored at -20°C until required.
- c. THYMIDINE-METHYL-<sup>3</sup>H 5'-TRIPHOSPHATE, tetrasodium salt (10-20 ci/mole) was purchased from New England Nuclear, Boston, Mass. Excess alcohol was removed either by flash-evaporation or by directing a gentle stream of nitrogen gas over the surface of the solution. The thymidine triphosphate concentration was adjusted to 0.1 mM by the addition of an appropriate quantity of "cold" thymidine triphosphate. The solution was stored at -20°C until required.
- d. TEMPLATES: Yeast RNA was obtained from Worthington, Freehold, N.J., and a stock solution of 1 mg/ml in distilled water was prepared and stored at -20°C until use. The other RNA templates, QBRNA, and 16S+23S rRNA from *E. coli*, were obtained from Miles Laboratories Inc., Elkhart, Indiana. Solutions of various concentrations (as indicated in the legends to the figures) were



prepared and stored at  $-20^{\circ}\text{C}$  until needed:

"Activated" calf thymus DNA was a gift from Dr. L.A. Loeb, prepared as described by Loeb (109) by subjecting the DNA to a limited digestion with pancreatic DNase until maximum priming ability with sea urchin DNA polymerase was obtained.

- e. "CARRIER" DNA: Calf thymus DNA purchased from Worthington or Sigma was stored at a concentration of 1 mg/ml in distilled water at either  $-20^{\circ}\text{C}$  or  $0-4^{\circ}\text{C}$ .
- f. RIBONUCLEASES: Both bovine pancreatic RNase A, and RNase  $T_1$  from *Aspergillus oryzae* were obtained from Sigma. Stock solutions of either 2 mg or 4 mg/ml, of pancreatic RNase A were prepared in distilled water, and heated for 10 minutes at  $95^{\circ}\text{C}$  to destroy any contaminating DNase. RNase  $T_1$  was used as an ammonium sulfate suspension at a concentration of 0.54 mg protein per ml.
- g. DEOXYRIBONUCLEASE I: Bovine pancreatic DNase I from Sigma, was prepared at a concentration of 1 mg/ml in water and stored at  $-20^{\circ}\text{C}$  until required.
- h. CLELAND'S REAGENT: Dithiothreitol (DTT) was purchased from Sigma, and a 0.2 M solution was prepared and stored at  $-20^{\circ}\text{C}$  until needed.
- i. INHIBITORS: Ethidium Bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) was purchased from Calbiochem, Los Angeles, California. Rifampicin (3-[4-methylpiperazinyl-imino-methyl]-rifamycin SV), actinomycin D, N-ethylmaleimide, and p-chloromercuribenzoic acid were from Sigma.

The rifamycin derivatives (for chemical nomenclature see Appendix I), rifamycin SV, AF/AP, AF/O13, AF/DNFI, AF/ABDP-cis, and M/14, were a gift of Dr. G. Lancini, Gruppo Lepetit, Milan, Italy. Solutions were freshly prepared in the appropriate solvents at the concentrations indicated in the legend to the appropriate figure.

- j. OTHER CHEMICALS: Trizma base (tris-[hydroxymethyl]aminomethane), EDTA (ethylenediamine-tetracetic acid), bovine serum albumin, dimethylsulfoxide, and deoxycholic acid were obtained from Sigma. Glycine was from Canaco, and maleic acid, perchloric acid, and sodium pyrophosphate were from J.T. Baker. Sucrose was obtained from the British Drug House, Toronto, Canada.

## (2) BUFFERS AND SOLUTIONS

- a. BUFFER A; Consists of 20 mM Tris-HCl, pH 8.0 (at 25°C), 40 mM KCl, 1 mM EDTA, and 0.5 mM DTT.
- b. BUFFER B; Consists of 20 mM glycine-NaOH, pH 9.0, 40 mM KCl, 1 mM EDTA, and 0.5 mM DTT.
- c. STOPPING SOLUTION; Consists of 1 M perchloric acid, and 0.05 M sodium pyrophosphate.
- d. WASHING SOLUTION; Consists of 0.5 M perchloric acid, and 0.025 M sodium pyrophosphate.
- e. MEDIUM A; (for the preparation of nuclei) Consists of 0.25 M sucrose in 0.05 M Tris-HCl, pH 7.5 (at 25°C), 0.025 M KCl, and 0.005 M MgCl<sub>2</sub> (TKM buffer).

f. MEDIUM B; (for preparation of nuclei) Consists of 2.3 M sucrose in TKM buffer.

g. ELECTROFOCUSING ELECTRODE SOLUTIONS:

LOWER ELECTRODE SOLUTION; Consists of 120 gm sucrose, 140 ml of distilled water, and 2 ml of concentrated phosphoric acid.

UPPER ELECTRODE SOLUTION: Consists of .1 percent NaOH in distilled water.

h. ELECTROFOCUSING GRADIENT SOLUTIONS:

DENSE GRADIENT SOLUTION; Consists of 30 ml glycerol and 1.8 ml, pH 3-10 ampholines (40% solution), made up to 50 ml with water.

LIGHT GRADIENT SOLUTION; Consists of 0.6 ml ampholine, pH 3-10 (40% solution) and 49.4 ml water..

B. METHODS

(1) PREPARATION OF TISSUE HOMOGENATES

Female Sprague-Dawley rats (100-150 gm) were first stunned and then sacrificed by cervical dislocation. The appropriate tissue was removed and quickly immersed in several volumes of ice-cold isotonic KCl solution. The tissue was then blotted, weighed, minced with scissors, and homogenized in three or four volumes, as indicated, of Buffer A. The homogenization was carried out in a Dounce glass homogenizer (Kontes Glass Company, Vineland, N.J.) using three strokes of the loose-fitting pestle (pestle A) followed by three strokes with pestle B. To enhance the extraction of DNA polymerase, the homogenate was further frozen and thawed twice, after which it was centrifuged at 39,000 g for 30 minutes in an International centrifuge. The 39,000 g supernatant served as the source of the enzymes examined.

## (2) PREPARATION OF THE RAT LIVER NUCLEI

Rat liver nuclei were prepared essentially by the method of Blobel and Potter (109). Rats were sacrificed as described above, the livers were excised and chilled immediately in several volumes of ice-cold Medium A. They were then blotted, weighed, and minced with scissors in two volumes of Medium A. This was followed by homogenization in a Potter-Elvehjem homogenizer with a motor-driven teflon pestle using 13-15 strokes. The homogenate was filtered through four layers of cheese cloth and 3 ml volumes were thoroughly mixed with 6 ml volumes of Medium B in polyallomer tubes that fit the SW 36 Spinco rotor. Consequently the sucrose concentration of the homogenate was raised to 1.62 M, yielding a density just sufficient to float mitochondria and rough endoplasmic reticulum. The mixture was then underlaid with 3 ml of Medium B using a syringe with a 13-gauge needle; the tip of the needle was placed at the bottom of the tube and the heavy sucrose solution introduced, forcing the lighter homogenate upward.

After centrifugation for 30 minutes at 35,000 rpm in a Spinco SW36 rotor, at 0-4°C, the supernatant was decanted and the material adhering to the walls of the tubes was removed with a spatula. The tube wall was then wiped dry with tissue paper, wrapped around a pair of forceps. The white nuclear pellet was taken up in 0.5 ml of either 0.14 M KCl for examination of the nuclei by phase contrast microscopy using a Carl Zeiss photomicroscope II (Carl Zeiss, Oberkochen, West Germany), or Buffer A for further fractionation and enzyme assay. Phase contrast microscopy was used as a criterion for the purity of the nuclear preparation.

(3) SEPHADEX CHROMATOGRAPHY

One ml of the 39,000 g supernatant of the tissue extract or 1 ml of the nuclear extract, treated as indicated in the legends, was applied to either a Sephadex G150 column (1.5 x 30 cm), equilibrated with Buffer A or to a Sephadex G200 column equilibrated with either Buffer A or Buffer B as indicated. The sample was eluted with the same buffer used to equilibrate the column, and fractions of either 0.5 or 1 ml, as indicated, were collected. Blue Dextran was used as a marker for determining the void volume. All accessories for Sephadex Chromatography were from Pharmacia Fine Chemicals, Montreal, Canada.

(4) ISOELECTRIC FOCUSING

Electrofocusing was carried out using the LKB 8101 column and LKB carrier ampholites, pH 3-10. The method differed from that described by Vesterberg and Svensson (107) in that the column was pre-equilibrated by applying a high voltage for 24 hours prior to the application of the sample. The column was set up as described in the LKB instruction manual.

The lower electrode solution (approximately 20 ml) was added into the lower electrode chamber. The density gradient (0-60% glycerol) with ampholines in the pH 3-10 range was prepared with a linear gradient mixer, using the light and dense solutions described in the section on "Buffers and Solutions". This was followed by the addition of sufficient upper electrode solution to reach a level of about one cm above the upper electrode. After pre-equilibration of the column for 24 hours, at 600-1000 volts, the sample (in 20% glycerol solution)

was inserted into the gradient through a thin tube. Equilibration was continued at 800-1000 volts for a further 18-24 hours, after which the contents of the column were collected from the bottom in 1.5 ml fractions by pumping distilled water through the top.

##### (5) ENZYME ASSAYS

###### a. RNA-DEPENDENT DNA POLYMERASE:

The assay for RNA-dependent DNA polymerase is similar to that reported by Spiegelman et al. (36), and Scolnick et al. (92). The standard reaction mixture consists of the following components in a total volume of 50  $\mu$ l.

RNA template (usually 1.2 mg/ml 16S + 23S rRNA)	10 $\mu$ l
RNA buffer mix	5 $\mu$ l
<div> <div>Tris-HCl (1M), pH 8</div> <div>dATP (5 mM)</div> <div>dCTP (5 mM)</div> <div>dGTP (5 mM)</div> <div>MgCl<sub>2</sub> (0.1 M)</div> </div>	<div>1.33 <math>\mu</math>l</div> <div>0.67 <math>\mu</math>l</div> <div>0.67 <math>\mu</math>l</div> <div>0.67 <math>\mu</math>l</div> <div>1.67 <math>\mu</math>l</div>
Dithiothreitol (0.2 M)	1 $\mu$ l
<sup>3</sup> H-dTTP (0.1 M)	2 $\mu$ l
"cold" dTTP (0.1 M)	2 $\mu$ l
distilled water	5 $\mu$ l
enzyme preparation	25 $\mu$ l

The assay consisted of incubating the reaction mixture (containing 25  $\mu$ l of the enzyme preparation) for 30 minutes, unless otherwise indicated, at 37°C. At the end of the incubation period, the reaction was stopped by first cooling the tubes in ice-water, followed by the addition of 1 ml Stopping Solution at 0°C (which precipitates the DNA product)

and 0.2 mg "carrier" DNA. The mixture was then vortexed, followed by centrifugation at 4,000 rpm for 15-30 minutes in a Sorvall RC 3 centrifuge with a swinging bucket rotor. The supernatant, containing the bulk of the unincorporated label, was then removed by aspiration. The pellet was dissolved in 1 ml 0.2 M NaOH at room temperature, and reprecipitated by the addition of 2 ml ice-cold stopping solution. The acid insoluble product was collected by vacuum filtration on a Whatman GF/C glass fiber disc, washed with three 2-3 ml aliquots of ice-cold distilled water, two 2-3 ml aliquots of washing solution followed by another two 2-3 ml aliquots of water. Finally, the filter disc was washed with an aliquot of 95% ethanol, dried under an infra-red heat lamp, and the radioactivity incorporated determined with a Beckman LS 233 liquid scintillation counter. The scintillation solution, consisted of 6 gm of scintillation grade PPO (2,5-diphenyl-oxazole) (Packard, Downers Grove, Illinois) per liter of toluene. Either glass scintillation counting vials from Packard, or disposable plastic vials from New England Nuclear were used.

b. ENDOGENOUS DNA-POLYMERASE ACTIVITY:

The assay for the endogenous activity was the same as that for the RNA-dependent DNA polymerase activity except that the RNA template was deleted and replaced by distilled water.

c. DNA-DEPENDENT DNA POLYMERASE:

The DNA-dependent DNA polymerase assay is based on a method described by Loeb (105). The standard reaction mixture consists of the following components, in a total volume of 50  $\mu$ l:

"Activated" calf thymus DNA (1.2 mg/ml)	5 $\mu$ l
DNA Buffer Mix	5 $\mu$ l
Tris-maleate (0.5 M) pH 7.4,	4.55 $\mu$ l
dATP (50 mM)	0.05 $\mu$ l
dCTP (50 mM)	0.05 $\mu$ l
dGTP (50 mM)	0.05 $\mu$ l
MgCl <sub>2</sub> (1 mM)	0.30 $\mu$ l
Dithiothreitol (0.2 M)	1 $\mu$ l
<sup>3</sup> H-dTTP (0.1 M)	2 $\mu$ l
distilled water	12 $\mu$ l
enzyme preparation	25 $\mu$ l

The rest of the assay was as described for the RNA-dependent DNA polymerase activity.

(6) PROTEIN DETERMINATION

Protein was measured by the method of Lowry et al. (108), using crystalline bovine serum albumin as standard.



## RESULTS

### (A) DETECTION AND PARTIAL PURIFICATION OF THE RNA-DEPENDENT DNA POLYMERASE ACTIVITY FROM RAT THYMUS

#### (1) ACTIVITIES IN THE SOLUBLE FRACTION OF THYMUS EXTRACT

The initial experiments, in attempting to detect an RNA-dependent DNA polymerase in mammalian tissues, were carried out with the 39,000 g supernatant of a 20% (w/v) rat thymus homogenate, using yeast RNA (yRNA) as the template (Fig. 1). The incorporation of radioactivity ( $^3\text{H-TMP}$ ) was proportional to the concentration of tissue extract at low concentrations (up to 5% w/v), after which the incorporation became non-linear, and in fact decreased at the highest concentration (20% w/v). Not all of the observed enzyme activity was dependent on added template (yRNA); that is, the endogenous activity (without added template, Fig. 1) accounted for about two-thirds of the activity observed in the presence of yRNA. For example, at a 4% w/v concentration of extract, 72% of the total activity was due to the endogenous template.

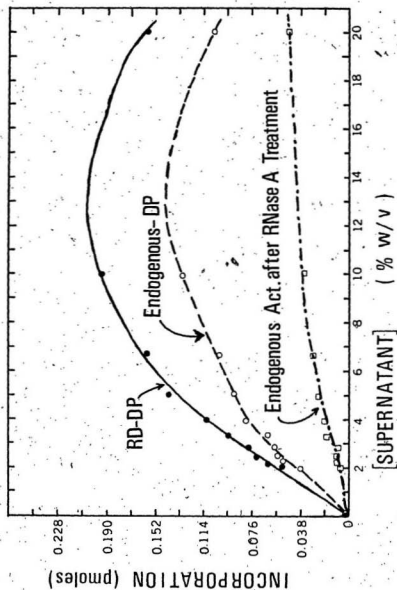
The endogenous RD-DPs from oncogenic RNA viruses (9, 10, 12), chicken embryos (77), uninfected rat cells (79), and human lymphocytes (80), are sensitive to RNase A. This was also the case with the endogenous activity from rat thymus. The results shown in Fig. 1 (bottom line) indicate that at least 77% of the endogenous activity in the 4% extract was sensitive to RNase A. The remaining activity is either due to a DNA template or else due to inefficiency on the part of RNase A itself in eliminating the endogenous template. The second possibility is supported by the observation that RNase A treatment is more efficient in eliminating

Fig. 1. RNA-DEPENDENT DNA POLYMERASE ACTIVITIES FROM RAT THYMUS.

A 20% (w/v) rat thymus homogenate was prepared in Buffer A as described in Methods. It was then centrifuged at 39,000 g for 30 minutes, in an International centrifuge and the supernatant was retained and used as the source of enzyme. Aliquots of supernatant were diluted to various concentrations (as indicated) by the addition of appropriate volumes of Buffer A. Reaction conditions for the RD-DP and endogenous activities were as described in Methods, with yRNA (5  $\mu$ gm/assay) as the template for the RD-DP. Unlabelled dTTP was not added to either the RD-DP or endogenous DNA polymerase assays (i.e.,  $^3$ H-TTP sp. act. was 4,750 cpm/pmole). The reaction mixture was then incubated for 15 minutes at 37°C, and the radioactivity incorporated was determined as described in Methods.

In the case of the RNase A treatment, 100  $\mu$ l of the enzyme preparation of various concentrations was treated with 10  $\mu$ gm of RNase A, for 30 minutes at room temperature, after which assays for the endogenous activity were repeated as above. All assays were carried out in duplicate, and the average plotted.

FIG. 1



the endogenous activity at the lower extract concentrations (Fig. 1). For example, in the case of a 2% extract, about 90% of the endogenous activity was eliminated, whereas an equal amount of RNase A eliminated only 77% of the activity in a 4% extract. These results could possibly indicate the presence of some factor or factors in the extract that interfere with the RNase A activity. For example, purine nucleoside di- and tri-phosphates (ADP, GDP, and ATP) are known to inhibit RNase A activity significantly (110). In addition, dithiothreitol, which is present in the homogenizing medium, has been shown to be a potent inhibitor of RNase A activity (111).

The problem associated with studying crude extracts indicated the necessity of looking for some purification method. Furthermore, the presence of the endogenous RNase-sensitive DNA polymerase (RS-DP) activity made it important to understand the relationship between this activity and the exogenous (RD-DP) activity. Therefore, an attempt was made to partially purify the enzyme activities by Sephadex gel filtration chromatography, before examining some of their properties.

## (2) GEL FILTRATION CHROMATOGRAPHY OF DNA POLYMERASES:

### (a) Endogenous activity -

Fig. 2 illustrates the Sephadex G 150 elution profile of the endogenous activity from a rat thymus extract. The enzyme eluted in the void volume, in the same fraction as Blue Dextran, suggesting a very high molecular weight. Furthermore, over 90% of this endogenous activity could be eliminated by treating the individual fractions with pancreatic RNase A (bottom curve). This higher sensitivity to the nuclease after

Fig. 2. SEPHADEX G 150 GEL FILTRATION CHROMATOGRAPHY OF THE RIBONUCLEASE-SENSITIVE DNA POLYMERASE FROM RAT THYMUS

A 25% (w/v) rat thymus homogenate was prepared and 1 ml of the 39,000 g supernatant was applied on a Sephadex G 150 column, equilibrated with Buffer A. The sample was eluted from the column with the same buffer, and 0.5 ml fractions were collected. Two 100  $\mu$ l aliquots were taken from each fraction, and to one set was added 10  $\mu$ l of a 50  $\mu$ g/ml RNase A solution, and to the other 10  $\mu$ l of distilled water to act as a control. They were then incubated for 30 minutes at room temperature and subsequently assayed for the endogenous activity as described for Fig. 1.

untreated (●—●)      RNase treated fractions (○—○)

Sp. act. of  $^3$ H-TTP was 4,750 cpm/pmole.

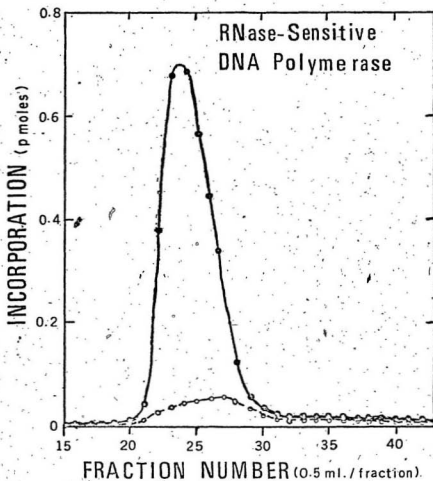


FIG. 2

gel filtration compared to the sensitivity in the crude extract, confirms the presence of some interfering factor which may have been removed through this fractionation procedure, and consequently rendered RNase A more efficient.

(b) *RNA- and DNA-dependent activities* -

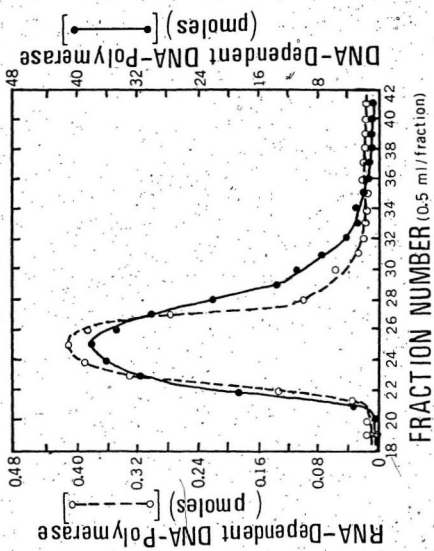
The finding of a DNA polymerase capable of copying an RNA template raises the question of whether this enzyme is physically the same as one of the known DNA-dependent DNA polymerases, or whether it is an enzyme with a unique specificity for RNA. To answer this question, an attempt was made to separate the two activities by gel filtration chromatography on a Sephadex G 150 column. The elution profile of the RNA- and DNA-dependent DNA polymerase activities is illustrated in Fig. 3. As can be seen from this figure, the peaks of activities coincide almost exactly suggesting that possibly only one enzyme is involved which can use both RNA and DNA as templates for DNA synthesis, although with much different efficiencies. These activities, however, eluted in the void volume, in a molecular weight range where Sephadex G 150 does not provide any resolution; hence this data does not indicate whether the activities are associated with the same enzyme species, or different enzymes. The observation that most of the RD-DP is associated with an endogenous template that is sensitive to RNase A (Fig. 2) suggests that this enzyme may be part of a nucleic acid-protein complex. Furthermore, judging from the known molecular weights for eukaryotic DD-DPs (112), it appears that the activity observed in the present experiments is associated with nucleic acids in a high molecular weight complex as has been reported for *E. coli* (113, 114) and PHA-stimulated normal human lymphocytes (85).

Fig. 3. PROFILE OF THE RD-DP and DD-DP ACTIVITIES FROM A RAT THYMUS  
EXTRACT FRACTIONATED ON A SEPHADEX/G 150 COLUMN

1 ml of a 25% rat thymus 39,000 g supernatant in Buffer A was fractionated on a Sephadex G 150 column, 0.5 ml fractions were collected and assayed for RD-DP as well as DD-DP activities. The RD-DP was assessed as described in the legend to Fig. 1. Reaction conditions for the DD-DP were as described in Methods, except that the reaction was carried out for 15 minutes at 37°C. 1 pmole of <sup>3</sup>H-TMP incorporated is equivalent to 4,900 cpm.



FIG. 3



### (3) EFFECT OF RIBONUCLEASE A TREATMENT:

#### (a) Initial experiments -

The possible occurrence of the endogenous RD-DP as a high molecular weight complex containing an internal RNA template suggested an experimental approach that might achieve two objectives. The approach which consisted of treating the thymus extract with RNase A prior to fractionation would presumably result in the destruction of the endogenous template, thereby converting the enzyme to a form capable of utilizing an externally added naturally-occurring RNA as template, and would also perhaps convert the activity to a low molecular weight form which could allow it to be separated from the major DNA-dependent DNA polymerase.

The thymus extract was therefore treated with either 50  $\mu\text{g}$ m (Fig. 4A) or 200  $\mu\text{g}$ m (Fig. 4B) pancreatic RNase A for 30 minutes at room temperature before fractionation. The profiles of the endogenous and RD-DP activities (Fig. 4) indicate that the RNase A treatment prior to fractionation eliminated part of the endogenous activity, although not as efficiently as the treatment after fractionation (Fig. 2). This is consistent with previous observations (Sections A 1, and A 2(a) of Results). Furthermore, the activity eluted in approximately the same region as the activity in the untreated extract (compare to Fig. 3). Hence the RNase A treatment only partially destroyed the endogenous template and was unsuccessful in releasing the enzyme from the high molecular weight complex.

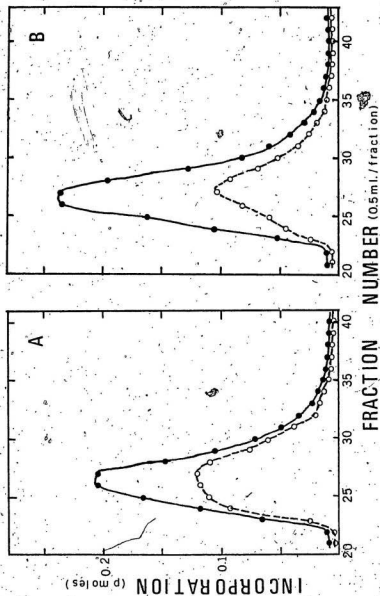
#### (b) Extensive RNase A treatment -

Since Sephadex G 150 gel filtration chromatography did not offer a good resolution in separating the activities, it was decided to fractionate

FIG. 4. SEPHADEX G 150 GEL FILTRATION CHROMATOGRAPHY OF THE RD-DP  
AND THE ENDOGENOUS ACTIVITY AFTER RNase A TREATMENT

1 ml. of the 39,000 g supernatant of a rat thymus homogenate was treated for 30 minutes at room temperature with either 50  $\mu$ gm (A) or 200  $\mu$ gm (B) of pancreatic RNase and subsequently fractionated on a Sephadex G 150 column. Activities were assessed as described in the legend to Fig. 1. 1 pmole of TMP incorporated is equivalent to 4,900 cpm.

RD-DP activity (● ————— ●)  
endogenous (○ - - - - ○)



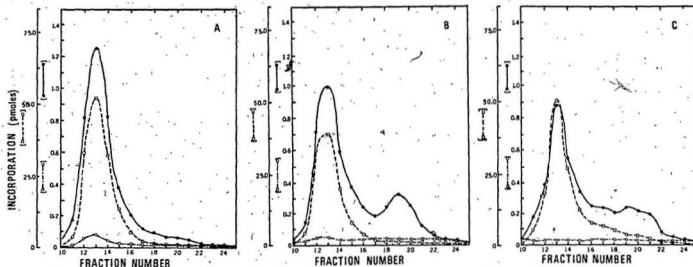
the enzymes on a Sephadex G 200 column after extensive RNase A treatments (Fig. 5). The profile of the DNA polymerases obtained from such a column when the thymus extract was not treated in any way (Fig. 5A) was similar to the result obtained with Sephadex G 150, except for the relatively low level of endogenous activity. With regard to this, I wish to mention that the level of this activity has been observed to vary considerably from preparation to preparation. Why this is so is not yet known, although physiological factors may be involved. The endogenous activity may also appear to be lower in this experiment, compared to the earlier studies because *E. coli* rRNA (which is more efficient than yRNA) was used as the template.

Prolonged RNase A treatment shifted part of the RD-DP activity to a lower molecular weight position on a Sephadex G 200 column (Fig. 5) but had almost no effect on the DP-DP activity. The profile of the activities after 3 or 6 hour treatments (Fig. 5, B and C respectively) is similar, although there is a more heterogenous distribution of the RD-DP activity after the 6 hour treatment, which may represent nucleic acid-enzyme complexes of various molecular weights being eluted from the column. The lack of a clearly defined peak may also be due to a loss of activity on the part of the enzyme. The amount of enzyme released has varied in different preparations from a ratio of 1 to 3 (of low MW to high MW enzymes), most often observed to as much as an equal distribution in both peaks. Doubling the amount of RNase A for the 3 hour treatment did not increase the amount of low MW RD-DP released.

If the thymus extract was allowed to stand at room temperature

FIG. 5

RELEASE OF RNA-DEPENDENT DNA POLYMERASE FOLLOWING PROLONGED RNase A TREATMENT



A 25% (w/v) rat thymus extract was prepared, and two 1 ml aliquots were treated with 200  $\mu$ m of pancreatic RNase A for 3 hours (B), and 6 hours (C) at room temperature followed by fractionation on a Sephadex G 200 column, equilibrated with Buffer A. A third 1 ml aliquot was fractionated on the same column without any treatment whatsoever (A). The enzyme activities were assayed as described in Methods, using 13.5  $\mu$ m per assay of 16S+23S rRNA as the template for RD-DP. The sp. act. for the RD-DP (●—●) and endogenous activity (○- -○) was 3,500 cpm per pmole, whereas that for the DD-DP (○- -○) was 6,000 cpm per pmole of  $^3$ H-TMP incorporated.

for three hours without any treatment whatsoever, the enzyme did not shift to the lower molecular weight position indicating that the shift is not merely a spontaneous dissociation process but rather is due to the hydrolysis of RNA associated with the enzyme. This conclusion is supported by the specificity of the effect of RNase A. The DD-DP activity was not noticeably shifted by this extensive RNase A treatment, suggesting the activities to be distinct. Furthermore, the concurrent loss of part of the endogenous activity, and the appearance of the low molecular weight RD-DP activity suggests that the two activities may be due to the same enzyme. The purity of this low molecular weight RD-DP is also two-fold (as determined by the Lowry et al. (108) method) higher than that of the high molecular weight enzyme and has a much lower DD-DP activity. The lack of any low MW peak of activity in the earlier experiment using Sephadex G 150 (Fig. 4) is probably due to the shorter time of RNase A treatment in that experiment (30 minutes) compared to the present experiment (3 hours).

(d) EFFECT OF RNase A PLUS RNase T<sub>1</sub> TREATMENT:

As reported above, pancreatic RNase A was successful in releasing only part of the RD-DP from the high to the low MW region. However, this RNase preferentially cleaves the pyrimidine ribonucleoside 3'-phosphate ester bond, and because of this specificity its action on RNA is limited. In fact, it has been claimed (115) that an undigested core rich in purine bases is formed during its action on RNA. For this reason an experiment was carried out whereby the thymus extract was treated not only with RNase A but also with RNase T<sub>1</sub> which is specific for breaking internucleotide bonds between 3'guanylic acid and 5'hydroxyl groups of adjacent nucleotides.

FIG. 6. SEPHADEX G 200 CHROMATOGRAPHY OF DNA POLYMERASES FROM RAT

THYMUS AFTER RNase A AND RNase T<sub>1</sub> TREATMENT

a 25% rat thymus homogenate was prepared in Buffer A as described in Methods and 1 ml was treated for 3 hours at room temperature in the presence of 400  $\mu$ g of RNase A and 27  $\mu$ g of RNase T<sub>1</sub>. This was followed by fractionation on Sephadex G 200. 1 ml fractions were collected, and assayed for RD-DP, endogenous DNA polymerase, and DP-DP activities as described in Methods. The specific activities were the same as described for Fig. 5.



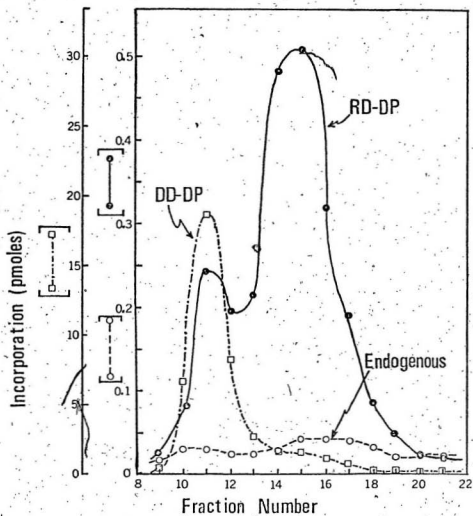


FIG. 6

(ibid p. 214). The results of this experiment (Fig. 6) indicate that a larger quantity of RD-DP activity can in fact be shifted to the lower MW region by the joint RNase A and  $T_1$  treatment, than by RNase A alone. (compare to Fig. 5). This further supports the conclusion that the low MW RD-DP activity is derived from the high MW activity.

(5) DNase I TREATMENT:

DNase I treatment had almost no effect on the endogenous RD-DP activity (Fig. 7), although the peak fraction of DD-DP was shifted toward a lower MW position than the peak fraction of the RD-DP. This does suggest that there are at least two distinct enzymes, although they are not well separated from each other's activity on this column.

Since prolonged RNase A treatment had been successful in releasing the RD-DP, a control experiment using extensive DNase I treatment was carried out (200  $\mu$ g/ml for 3 hours). A large amount of precipitate was formed during this treatment, which was necessary to remove by centrifugation before applying the extract to the Sephadex column. It was found that almost 90% of the DD-DP activity was lost although a lower proportion of RD-DP activity had been lost (Fig. 8B). This phenomenon can be explained in several ways; either the enzymes were precipitated and/or inactivated during the DNase I treatment or some of the DNase I may have adsorbed to the high MW complex and destroyed a large portion of the product formed. In a control experiment (Fig. 8A) an aliquot of the same extract was treated with an equal quantity of  $MgCl_2$  as used in the DNase I treatment. In this case a smaller quantity of precipitate was observed, and on fractionating the supernatant the usual levels of activity were recovered,

FIG. 7. SEPHADEX G 150 GEL FILTRATION CHROMATOGRAPHY OF THE RD-DP, ENDOGENOUS DNA POLYMERASE AND DNA-DEPENDENT DNA POLYMERASE AFTER DNase TREATMENT

a 25% rat thymus extract was prepared as described in Methods. 1 ml was treated with 25  $\mu$ gm of DNase I and 50  $\mu$ l of a 0.1 M  $MgCl_2$  solution, for 30 minutes at 37°C. It was then fractionated on a Sephadex G 150 column and 0.5 ml fractions were collected and assayed for RD-DP (  $\square$  - - -  $\square$  ), endogenous DNA polymerase (  $\circ$  - -  $\circ$  ), and DD-DP (  $\bullet$  —  $\bullet$  ). Reaction conditions were identical to those described in the legend to Fig. 1 (for the RD-DP and endogenous activities) and in Methods (for the DD-DP). 1 pmole of TMP incorporated is equivalent to 4,900 cpm.

FIG. 7

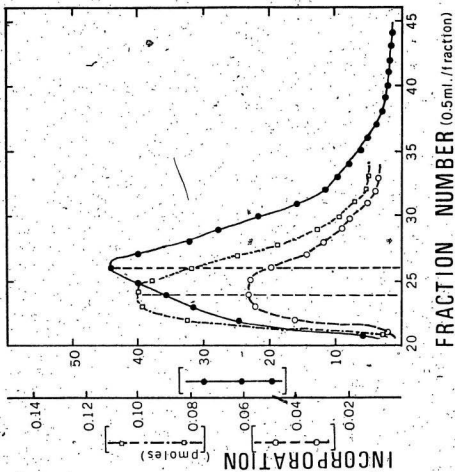
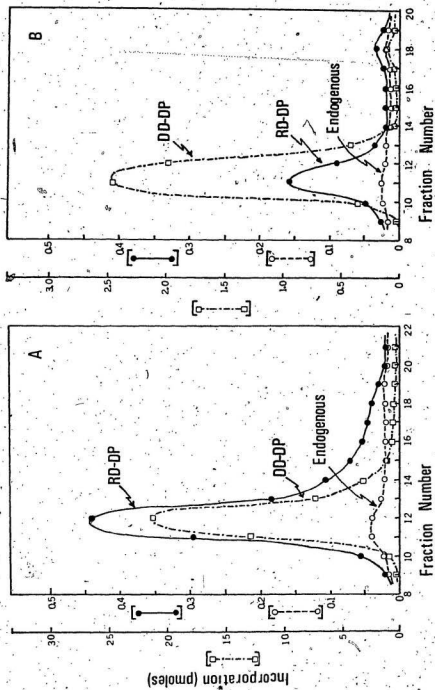


FIG. 8. SEPHADEX G 200 GEL FILTRATION CHROMATOGRAPHY OF THE DNA  
POLYMERASES FROM RAT THYMUS AFTER AN EXTENSIVE DNase I  
TREATMENT

A 25% (w/v) rat thymus homogenate was prepared in Buffer A as described in Methods. (A) 1 ml of the extract was incubated at room temperature in the presence of 20 mM  $MgCl_2$  for 3 hours. It was then centrifuged at 2,500 g for 10 minutes to remove any precipitated material, and the supernatant fractionated on a Sephadex G 200 column. (B) 1 ml of the extract was treated with 200  $\mu$ gm of DNase I in the presence of 20 mM  $MgCl_2$  for three hours at room temperature. The large quantity of precipitate formed was then removed and the sample fractionated as described above. Activities were assessed as described in Methods. The sp. act. of  $^3H$ -TTP for the RD-DP and endogenous reaction mixtures was 3,625 cpm/pmol and that for the DD-DP 6,000 cpm/pmol. Note the 10-fold difference in scales for the DD-DP activity between Figs. A and B.

FIG. 8



indicating that DNase I and not the increase in ionic strength had been responsible for the loss of activity. A comparison of the levels of RD-DP and DD-DP activities recovered under the two treatments, shows that approximately 88% of the DD-DP activity was lost during DNase I treatment compared to 69% of the RD-DP activity. This differential loss of activity is consistent with the idea that different enzymes are responsible for the two activities.

## B) DISTRIBUTION AND SUBCELLULAR LOCALIZATION OF DNA POLYMERASES FROM RAT TISSUES

### (1) RELATIVE ACTIVITIES IN VARIOUS RAT TISSUES

It is clear from the data presented until now that an RNA-dependent DNA polymerase activity is indeed present in rat thymus. Previous reports in the literature have indicated the activity to be present in a variety of systems such as PHA-stimulated human lymphocytes (72, 73), rat liver (100), *E. coli* (81-83), and chick embryos (77), which have been discussed in the *Literature Review*.

Having available an apparently reliable and sufficiently sensitive assay system for the RD-DP, it was decided to screen various rat tissues to determine the distribution of this activity in the rat. The results of this study (Fig. 9) indicate that all of the tissues examined contain the activity. Table I lists these tissues in order of decreasing amounts of RD-DP activity present in each (based on the activity found in thymus) and also indicates the amount of soluble DD-DP obtained from the individual tissues, under the same preparative conditions. These results leave little doubt in regard to the ubiquitous distribution of RD-DP in rat tissues.

FIG. 9. DNA POLYMERASES FROM VARIOUS RAT TISSUES

25% (w/v) rat tissue homogenates were prepared in Buffer A as described in Methods, and 1 ml of each of the 39,000 g supernatants were fractionated separately on a Sephadex G.150 column equilibrated with the same buffer. The RD-DP, endogenous, and DD-DP activities were assessed as described in Methods using rRNA as the template for the RD-DP. The sp. act. of  $^3\text{H}$ -TTP for the RD-DP, and endogenous activities was 3,625 cpm/pmole and that for the DD-DP, 6,000 cpm/pmole.

● — ● rRNA-dependent activity  
○ - - - ○ endogenous  
□ - - - □ DD-DP



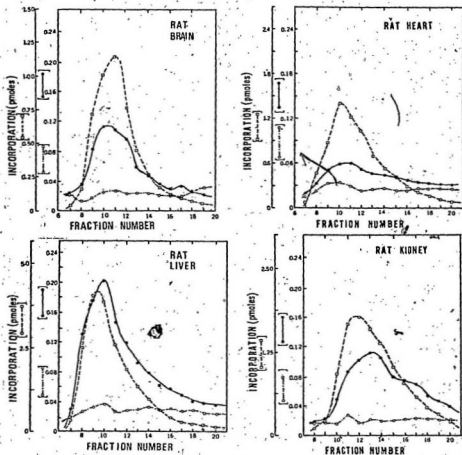


FIG. 9

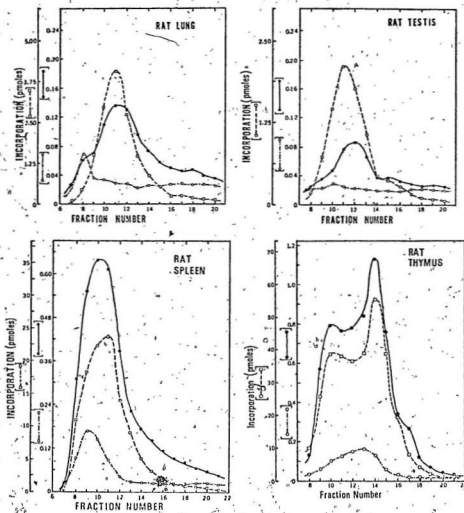


FIG. 9 (CONTD.).

TABLE I

RELATIVE ABUNDANCE OF SOLUBLE RD-DP AND DD-DP ACTIVITIES  
FROM VARIOUS RAT TISSUES

<u>Tissue</u>	<u>% Activity</u>		<u>Ratio (DD/RD)</u>
	<u>RD-DP</u>	<u>DD-DP</u>	
Thymus	100 <sup>1</sup>	100 <sup>2</sup>	53.4
Spleen	69.7	39	30.2
Brain	35.8	10.3	15.2
Liver	25.6	6.3	13.0
Lung	23.5	6.7	14.8
Kidney	17.3	4.3	13.3
Testis	11.7	3.8	16.8
Heart	6.7	1.8	13.9
Plasma	2.8	0.003	0.05
Red Blood Cells	2.4	0.002	0.06

The values are based on the average of all the active fractions eluting from the Sephadex G 150 column (Fig. 9), after subtracting the background.

<sup>1</sup> 100% activity is equivalent to 0.58  $\mu$ moles of TMP incorporated per assay, the specific activity being 3,625 cpm per  $\mu$ mole.

<sup>2</sup> 100% activity is equal to 31  $\mu$ moles of <sup>3</sup>H-TMP incorporated, the specific activity being 6,000 cpm/ $\mu$ mole.

TABLE II

RELATIVE ABUNDANCE OF RD-DP AND DD-DP ACTIVITIES IN VARIOUS  
RAT TISSUES AFTER STORAGE FOR VARIOUS LENGTHS OF TIME

<u>Tissue</u>	<u>DD-DP / RD-DP</u>			
	<u>Number of weeks stored at -20°C</u>			
	<u>1</u>	<u>4</u>	<u>6</u>	<u>10</u>
Liver	16.6	6.5	7.2	6.3
Spleen	45.1	19.8	12.9	9.5
Lung	29.9	19.4	15.1	9.8
Kidney	15.2	9.5	7.2	8.0
Brain	4.5	1.6	2.2	3.9
Testis	13.4	5.6	4.8	6.7
Heart	16.1	4.6	4.5	12.4

All of the above values were based on the data from Fig. 10 after converting cpm to pmoles incorporated.

Penner et al. (72, 93) have previously shown that the level of RD-DP activity increases during PHA-stimulation of cultured human lymphocytes from normal patients. The low level of activity in the plasma of rat may be explained by the fact that the cells are in a quiescent state.

In most tissues the profiles of the RD-DP and DD-DP from a Sephadex G-150 column coincided to a large extent (Fig. 9), although the ratios of these activities varied widely (Table I).

(2) STABILITY OF RD-DP AND DD-DP AT -20°C and ACTIVATION OF RD-DP UPON FREEZING

The next experiments were directed towards determining the stability of the two activities in some of the tissues examined at -20°C. A differential stability was detected for the two activities (Fig. 10). Assays were carried out after 1, 4, 6, and 10 weeks of storage. In the case of the RD-DP an increase in activity was observed for all tissues except the lung, at the fourth week of storage, as compared to the first week. After four weeks of storage the activity began to decrease. In the case of the DD-DP no activation at all was observed; and the enzyme lost activity at a faster rate than the RD-DP.

Table II compares the ratios of the level of DD-DP to the RD-DP activities remaining after various lengths of time. The ratio between the two activities has varied from as high as 45.1:1 in spleen to as low as 1.6:1 in brain. Ratios of about 200:1 have been observed in the case of the thymus (data not shown in Table II).

In one particular experiment conducted with the thymus extract, it was observed that a four-fold stimulation of both the endogenous and

FIG. 10. STABILITY WITH STORAGE OF DNA POLYMERASES FROM RAT TISSUES

The most active fractions from some of the gel filtration experiments shown in Fig. 9 were pooled and stored at  $-20^{\circ}\text{C}$  for various lengths of time, after which the RD-DP and DD-DP activities were assessed under the conditions described in Methods. Reactions were carried out for 30 minutes at  $37^{\circ}\text{C}$  as described in Methods. 100% activity in terms of cpm (after subtracting background) for the various tissues are listed below:

	<u>RD-DP</u>	<u>DD-DP</u>
Liver	528	14,604
Spleen	1435	107,157
Lung	484	23,936
Kidney	356	8,971
Brain	738	5,444
Testis	241	5,360
Heart	133	3,546

Sp. act. of  $^3\text{H}$ -TTP for the two reaction mixtures were as described for Fig. 9.

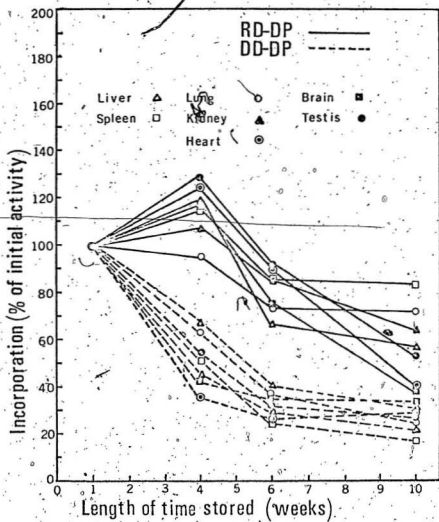


FIG. 10

RD-DP activities occurred upon overnight storage of the enzyme preparation at  $-20^{\circ}\text{C}$  (data not shown) although as a rule smaller activations were observed. The significance of these results is unclear, although several explanations do come to mind. One possibility is that the enzyme undergoes a conformational change upon freezing. Alternately, the activation may be due to loss of an inhibitor of the polymerase, which may occur during storage.

### (3) SUBCELLULAR LOCALIZATION OF DNA POLYMERASES FROM RAT LIVER

Having established the presence of RD-DP in most rat tissues, it became of interest to determine where the activity was actually located within the cell; specifically, whether it was of cytoplasmic or of nuclear origin. Such an investigation would perhaps indirectly make available an enzyme preparation more free of DD-DP and in addition the enzyme's location might suggest something with regard to its function. Rat liver was used in this study for several reasons: first, an isolation procedure for obtaining very clean nuclei from this tissue was available (106), and second, rat liver has a relatively low level of DD-DP activity.

Phase contrast microscopy was used for identifying the nuclei and showing their purity (Fig. 11). As can be seen from this micrograph, the preparation consists essentially of nuclei, some of which (a very low percentage) appear to be broken. The activities of the DNA polymerases in the nuclear and cytoplasmic fractions as well as in the crude liver homogenate were assessed (Table III). The various activities have been expressed in terms of total units (pmoles of TMP incorporated) of activity as well as in terms of specific activity (units/mg of protein). The specific activity of the RD-DP activity in the nuclear fraction was about



FIG. 11. PHASE CONTRAST MICROSCOPY OF RAT LIVER NUCLEI

Rat liver nuclei were prepared as described in Methods. The nuclei obtained from a 3 ml 33.3% (w/v) liver homogenate were taken up in 0.5 ml of isotonic KCl and examined and photographed by phase contrast microscopy using a Carl Zeiss photomicroscope II.

Magnification factor X 1,230

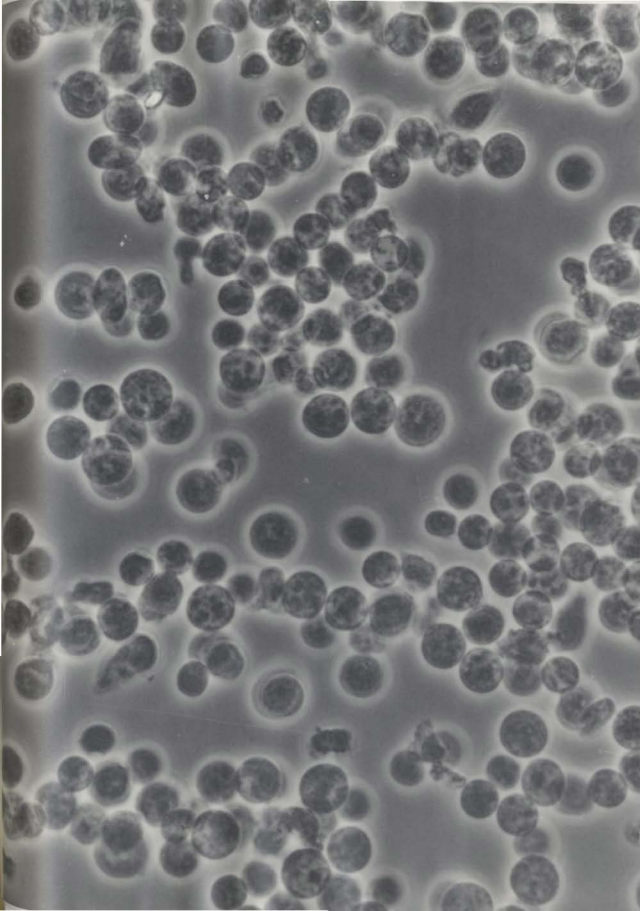


TABLE III

## NUCLEAR AND CYTOPLASMIC DISTRIBUTION OF DNA POLYMERASES FROM RAT LIVER

Activity	Fraction	Total Units <sup>1</sup>	Units mg prot.	% Yield
RD-DP	Crude extract	22.8	3.9	100
	Cytoplasmic fraction	14.2	1.3	61.8
	Nuclear fraction	2.3	4.8	10.1
Endogenous	Crude extract	14.7	2.5	100
	Cytoplasmic fraction	8.2	0.8	55.8
	Nuclear fraction	2.0	4.2	13.6
DD-DP	Crude extract	48.5	8.3	100
	Cytoplasmic fraction	118.0	21.9	243.3
	Nuclear extract	16.1	35.3	33.2

Rat liver nuclei were prepared as described in Methods, by the method of Blobel and Potter (106). The nuclear fraction, the cytoplasmic fraction, and the crude extract were assayed for RD-DP, endogenous and DD-DP activities as described in Methods, after making appropriate dilutions with Buffer A to assure that the activities were assessed in a range where it was proportional to the enzyme concentration. The crude extract represents the filtrate obtained after filtering the homogenate through cheese cloth. Proteins were estimated by the method of Lowry et al. (108). The specific activity for the endogenous and RD-DP was 3,625 cpm/pmole of TMP incorporated, and that for the DD-DP, 6,000 cpm/pmole.

<sup>1</sup> 1 unit is equal to 1 pmole of TMP incorporated.

3.7 times as high as in the cytoplasmic fraction; however, in terms of total activity there appears to be 7 times as much in the cytoplasm as in the nuclear supernatant. Both the endogenous and DD-DP activities displayed parallel results, although the ratios in the two fractions were not identical to those for the RD-DP.

Some experiments conducted in the presence of insoluble material (i.e., membrane fragments), after freezing and thawing the nuclei, revealed much higher levels of DNA polymerase activities as compared to the levels remaining after sedimenting this material (Table IV). Hence, the next experiments were directed towards attempting to solubilize the activities associated with this insoluble material, and deoxycholate (DOC) was used as the solubilizer.

Fig. 12 shows the amount of DNA polymerases solubilized by various concentrations of DOC. Concentrations above 0.25% of the detergent caused coagulation. The supernatants of the nuclear preparations treated with various quantities of detergent were assayed for the activities after diluting to 1/5 the original concentrations. Substantial amounts of the activities were found to be solubilized by 0.25% (w/v) of the detergent. The soluble RD-DP was increased more than 10-fold, the endogenous activity more than 8-fold, and the DD-DP more than 5-fold. These results indicate that the level of the activities within the nucleus is higher than reported in Table III. As to whether the activities are associated with the nuclear membrane, or with rapidly sedimenting nucleoprotein complexes is not known.

TABLE IV

## DNA POLYMERASE ACTIVITIES IN RAT LIVER NUCLEI

	<u>RD-DP</u>	<u>Endogenous</u>	<u>DD-DP</u>
Total nuclei <sup>1</sup>	883	505	6,093
Nuclear supernatant <sup>2</sup>	341	300	4,193

Rat liver nuclei were prepared, and activities assessed, as described in "

Methods. Specific activities were 3,625 cpm per pmole of TMP incorporated for the RD-DP and endogenous activities, and 6,000 cpm for the DD-DP.

<sup>1</sup> Nuclei were frozen and thawed twice, and the activities assessed in the presence of insoluble material.

<sup>2</sup> The frozen and thawed nuclei were centrifuged at 7,000 g for 20 minutes and the supernatant used for assays.

FIG. 12. SOLUBILIZATION OF DNA POLYMERASES FROM RAT LIVER NUCLEI BY  
DEOXYCHOLATE

Rat liver nuclei were prepared as described in Methods, and the nuclei from 18 ml of liver homogenate were taken up in 3 ml of Buffer A. 360  $\mu$ l aliquots were then treated with various concentrations of deoxycholate in final volumes of 400  $\mu$ l, for 15 minutes at 0 - 4°C, after which they were centrifuged at 7,000 g for 20 minutes. The supernatants were then diluted to 1/5 the original concentration and the RD-DP, endogenous, and DD-DP activities assessed in duplicate as described in Methods. The specific activity for the RD-DP and endogenous activities was 3,625 cpm/pmole of TMP incorporated and that for DD-DP, 6,000 cpm/pmole.

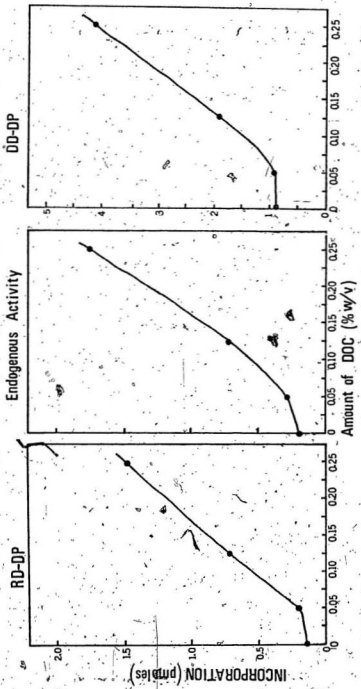


FIG. 12

(C) CHARACTERIZATION OF DNA POLYMERASES FROM RAT THYMUS

The results of the distribution study (Fig. 9 and Table I) showed that the thymus had the greatest amount of RNA-dependent DNA polymerase per gram of tissue. The thymus was therefore selected for further characterization of the activity.

(1) ISOELECTRIC POINTS OF THE DNA POLYMERASES

Electrofocusing is a high resolution protein separation technique which operates on a different principle than gel filtration chromatography. This approach was therefore used, both to obtain the isoelectric points of the enzymes being studied, as well as to possibly separate the different activities. The electrofocusing profile of the DNA polymerases from rat thymus (Fig. 13) shows one main peak containing both RNA- and DNA-dependent DNA polymerase activities at an isoelectric point of 4.6. In addition, the RD-DP also gave a series of other peaks with a main one at an isoelectric point of 5.3. Whether these various peaks represent different isozymes or different enzyme-nucleic acid complexes is not known. It should be noted, however, that the electrofocusing results closely parallel those obtained with Sephadex chromatography.

(2) MOLECULAR WEIGHT DETERMINATION OF THE DNA POLYMERASES

Fig. 14 (top) shows the elution profile of the DNA polymerases from a Sephadex G 200 column. The column was calibrated with several proteins of known MW [RNase A (13,700), ovalbumin (45,000), and aldolase (158,000)], (Fig. 14, bottom) allowing an approximate MW of 120,000 to be estimated for the low MW RD-DP. The size of the high MW complex cannot be obtained from this data because it elutes very near the void volume.



FIG. 13. ISOELECTRIC FOCUSING OF THE DNA-POLYMERASES FROM RAT THYMUS

Isoelectric focusing was carried out in the pH 3 - 10 range, as described in Methods. A 20% (w/v) thymus extract in Buffer A was prepared and to 1.2 ml were added 250  $\mu$ l of glycerol before it was introduced into the column. Focusing was carried out for approximately 24 hours, after which 1.5 ml fractions were collected and assayed for the RD-DP (□-----□) and endogenous (o - - o) activity as described in the legend to Fig. 1, and the DD-DP (●-----●) as described in Methods. Reactions were carried out for 15 minutes at 37°C. The specific activity was 3,500 cpm/pmole of TMP incorporated.

FIG. 13

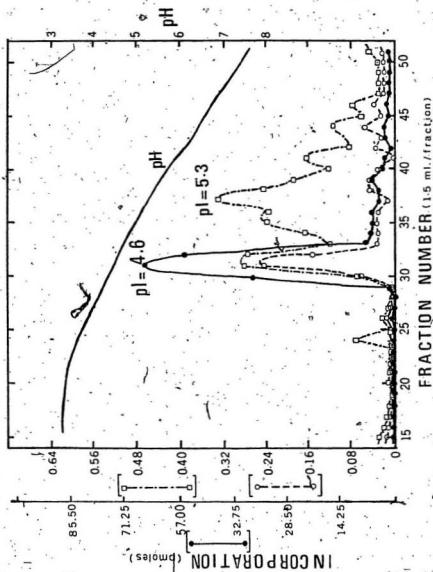


FIG. 14. MOLECULAR WEIGHT DETERMINATION OF THE RD-DP ACTIVITY ELUTING IN  
THE LOW MOLECULAR WEIGHT REGION OF A SEPHADEX G 200 COLUMN

1 ml of a 25% thymus extract was treated for 3 hours at room temperature with 400  $\mu$ g of RNase A and subsequently fractionated on the same column. The activities (RD-DP, endogenous, and DD-DP) were determined as described in Methods. The MW of the low MW RD-DP estimated from the calibration curve (lower graph) was approximately 120,000. The MW of the other activities could not be determined since they eluted in the void volume.

A Sephadex G 200 column was calibrated using standard proteins of known molecular weights such as RNase A (MW 13,700), ovalbumin (MW 45,000) and aldolase (MW 158,000), each at a concentration of 1 mg per ml. Their elution position was determined spectrophotometrically by reading the absorbance of each fraction at 280 nm.

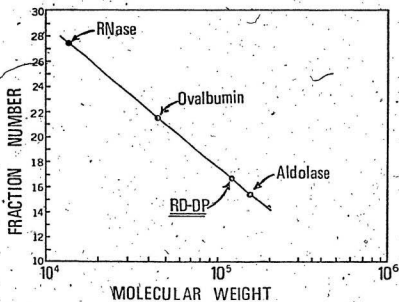
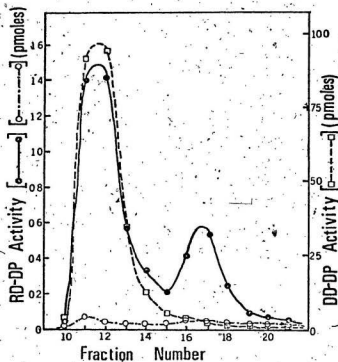


FIG. 14

### (3) TIME-DEPENDENCE OF THE ENDOGENOUS AND RD-DP ACTIVITIES:

#### (a) Endogenous activity -

Fig. 15 illustrates the time dependence of the RS-DP or endogenous DNA polymerase activity at two different temperatures ( $30^{\circ}$  and  $37^{\circ}\text{C}$ ), and demonstrates the reaction to be nonlinear after the first ten minutes. This nonlinearity may be due to one or more factors, the most likely being depletion of the template itself. It is not likely that the substrates (deoxynucleoside triphosphates) are being depleted since they were present at high concentrations and also, in other experiments with a DNA template, at least 100-fold greater incorporation was obtained, using similar substrate concentrations (see Fig. 3). Another possibility is that the enzyme preparation was still contaminated with some type of nuclease responsible for the breakdown of either the template or the product. In any case the non-linearity of the reaction has made the characterization of this activity difficult.

#### (b) rRNA-dependent reaction -

The rRNA-dependent DNA polymerase reaction from the low MW region is linear with time for at least 30 minutes (Fig. 16) after which the rate decreases slightly, but the amount of incorporation continues to increase within the range of time examined (60 minutes). Taking these data together with those in the preceding experiment (Fig. 15, time-dependence of the endogenous activity) in which reaction conditions were identical except for the absence of exogenous template, we can conclude that the lack of linearity for the endogenous activity is due to insufficient endogenous template.

FIG. 15. TIME-DEPENDENCE OF THE RIBONUCLEASE-SENSITIVE DNA POLYMERASE  
ACTIVITY FROM RAT THYMUS EXTRACT PARTIALLY PURIFIED ON A  
SEPHADEX G 150 COLUMN

The supernatant of a 25% (w/v) rat thymus extract was  
fractionated as described in the legend to Fig. 2. The most active  
fractions were pooled and assays for the endogenous activity were  
then repeated in duplicate for various incubation times at two  
temperatures (37° top, 30° bottom). Sp. Act. of <sup>3</sup>H-TTP was 3,500  
cpm/pmole.

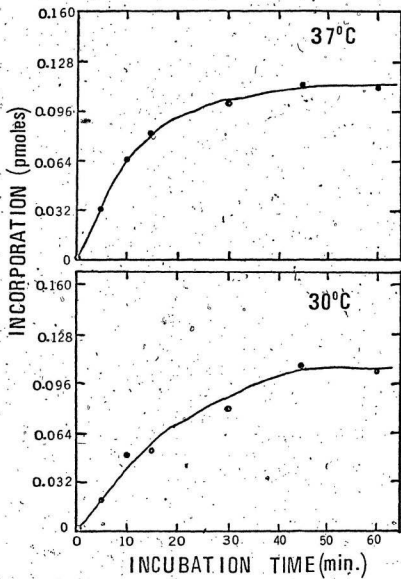


FIG. 15

FIG. 16. TIME-DEPENDENCE OF THE rRNA-DEPENDENT DNA POLYMERASE

The low MW RD-DP eluting from a Sephadex G200 column was used as the source of activity. Reaction conditions were as described in Methods with 13.5  $\mu$ gm 16S + 23S rRNA per assay, and "cold" dTTP deleted. The reactions were incubated for various lengths of time at 37°C and each point represents the average of duplicates. The specific activity of  $^3$ H-TTP is 7,140 cpm/pmole.



RD-DP

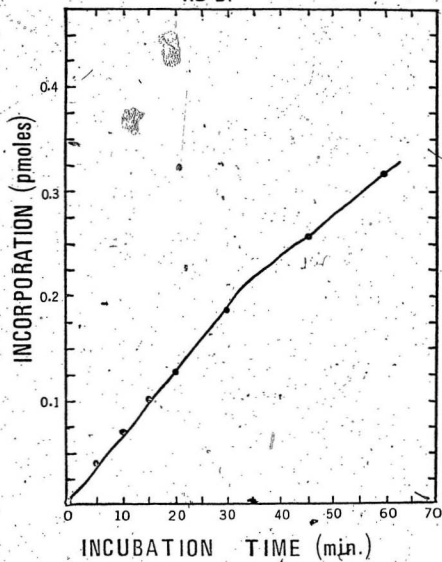


FIG. 16

#### (4) EFFECT OF ENZYME CONCENTRATION ON INCORPORATION

The effect of enzyme concentration on the velocity of the reaction (Fig. 17) displays a sigmoidal relationship for the RD-DP, suggesting the possibility of a subunit structure for the enzyme. On the other hand, the DD-DP activity was found to be linear up to 25  $\mu$ l of the enzyme preparation, after which it plateaued. Assays were normally carried out in the linear range.

#### (5) REQUIREMENTS OF THE ENDOGENOUS AND RD-DP ACTIVITIES:

##### (a) Endogenous activity -

The requirements of the endogenous activity are shown in Table V. As expected for a DNA polymerase, maximum activity depends on the presence of all four deoxynucleoside triphosphates. The deletion of all three unlabelled deoxynucleoside triphosphates results in approximately one-ninth of the activity observed in their presence, while the deletion of each individually is less effective. Pancreatic RNase A treatment was found to eliminate 95% of the activity, suggesting that an RNA template is required for the endogenous reaction. An absolute requirement for a divalent cation was evident. Both  $Mg^{++}$  and  $Mn^{++}$  were used by the enzyme although with different efficiencies.  $Mn^{++}$ , at a concentration one-tenth that of  $Mg^{++}$ , proved to be twice as efficient as  $Mg^{++}$ . Since less reproducible results were obtained with  $Mn^{++}$ ,  $Mg^{++}$  was preferred to  $Mn^{++}$  in carrying out any further experiments. This irreproducibility with  $Mn^{++}$  as the activator is consistent with the findings of other workers (100).

The need for all four deoxynucleoside triphosphates for maximum activity suggests that a terminal nucleotidyl transferase similar to the

FIG. 17. ENZYME CONCENTRATION CURVES FOR THE RD-DP AND DD-DP  
ACTIVITIES FROM RAT THYMUS

The most active fractions from the low MW peak were used for the RD-DP and the active fractions eluting in the high MW region for the DD-DP. Assay conditions were as described in Methods, except that the amount of enzyme preparation was varied. All assays were carried out in duplicate and incubated for 30 minutes at 37°C. The specific activity of  $^3\text{H}$ -TTP was 3,625 cpm/pmole for the RD-DP, and 6,000 cpm/pmole for the DD-DP.

FIG. 17

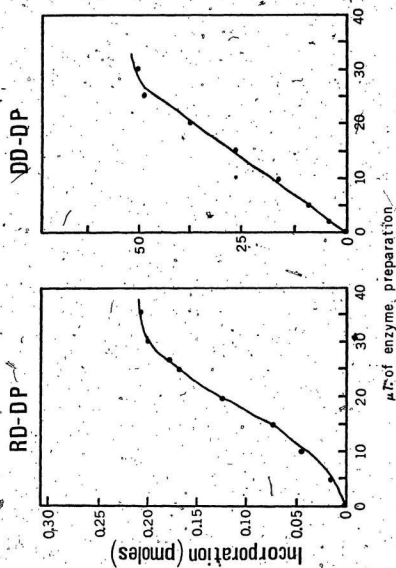


TABLE V

## REQUIREMENTS OF THE RIBONUCLEASE-SENSITIVE DNA POLYMERASE

## OF RAT THYMUS

Reaction Mixture	Incorporation of $^3\text{H-TMP}$ (%)
Complete <sup>1</sup>	100
-dATP, -dCTP, -dGTP	11.67
-dATP	28.60
-dCTP	45.67
-dGTP	47.76
Complete <sup>2</sup>	80.61
+RNase A <sup>3</sup>	5.17
Divalent cation requirement:	
Complete <sup>4</sup>	100
-Mg <sup>++</sup>	0
-Mg <sup>++</sup> , +Mn <sup>++5</sup>	212.87

The enzyme preparation was obtained as described in the legend to Fig. 2. The reactions were carried out as described in the legend to Fig. 1 under the conditions denoted in the first column. A background of 70 cpm has been subtracted.

<sup>1</sup>100% activity is equivalent to 1,388 cpm/assay.

<sup>2</sup>Water was added (10  $\mu\text{l}$   $\text{H}_2\text{O}$ /100  $\mu\text{l}$  enzyme preparation) and the enzyme preparation incubated 30 minutes at room temperature to serve as a control for RNase treatment.

<sup>3</sup>RNase A treatment [10  $\mu\text{l}$  RNase A (2 mg/ml) per 100  $\mu\text{l}$  enzyme preparation] for 80 minutes at room temperature.

<sup>4</sup>100% activity is equivalent to 893 cpm/assay.

<sup>5</sup>Mg <sup>++</sup> (final concentration of 5 mM) was replaced by 0.5 mM Mn <sup>++</sup>.

one reported in calf thymus (116), is not the activity that is being detected and furthermore, an heterogeneous region, and not a poly A region of a template sensitive to RNase A, is being copied.

(b) *rRNA-dependent DNA polymerase activity-*

Table VI shows the requirements of the low MW RD-DP as well as some of the properties of the product and the template. All four deoxynucleoside triphosphates are required for maximum activity. Deletion of dATP, dCTP, and dGTP, resulted in only 13.3% of the activity observed in the presence of all four deoxynucleoside triphosphates, while deletion of each one individually resulted in a smaller decrease in activity. If the template rRNA is treated with RNase A prior to carrying out the assay, 66.8% of the maximum activity is recovered. However, on being treated with both RNase A and  $T_1$ , the activity decreases to only 13.4% of the maximum, indicating that RNA is necessary for enzyme activity. Leaving the template out of the reaction pool, yielded only 4.3% of the maximum activity. If DNase I was added to the reaction mixture after 30 minutes, no incorporation was observed, indicating the product to be DNA. Furthermore, the product was stable to RNase A, added after 30 minutes and further incubating for another 30 minutes (89.4% remained of the activity observed after a one hour incubation). Assays for the DD-DP activity in the same fractions as the RD-DP yielded 36.96 times as much activity as the RD-DP. As to whether this represents the activity of a contaminating enzyme or whether the RD-DP enzyme also uses DNA as a template is still not known, although there have been certain preparations in which the ratio of RD-DP to DD-DP has been as high as 1 to 5, suggesting that at least some of the DNA directed activity is due to a contaminating enzyme.

TABLE VI

## REQUIREMENTS OF THE RNA-DEPENDENT DNA POLYMERASE FROM RAT THYMUS

Reaction conditions	Incorporation of <sup>3</sup> H-TMP (%)	
Experiment I -		
Complete <sup>1</sup>	100	
-dATP, -dCTP, -dGTP	13.3	
-dATP	35.9	
-dCTP	52.8	
-dGTP	42.0	
-RNA	4.4	
RNase A treated rRNA <sup>2</sup>	66.8	
RNase A + RNase T <sub>1</sub> treated rRNA <sup>3</sup>	13.4	
+ "activated" calf thymus DNA <sup>4</sup>	3696.0	
Experiment II -		
Complete <sup>5</sup>	100	
+ DNase I (introduced at 30 minutes) <sup>6</sup>	0	
+ RNase A (introduced at 30 minutes) <sup>7</sup>	89.4	
Experiment III -		
Complete <sup>8</sup>	100	
heated rRNA <sup>9</sup>	72.2	100
DNase I treated rRNA <sup>10</sup>	—	39.5

The low molecular weight RD-DP peak was used for all assays. Reactions were carried out under the conditions denoted in the first column, and incubated for 30 minutes in the case of Experiments I and III, and for

TABLE VI (Contd.)

one hour in the case of Experiment II. A background of 60 cpm has been subtracted from all values.

- 1 Reaction conditions were as described in Methods. 100% activity is equivalent to 773 cpm.
- 2 100  $\mu$ l of a 1.49 mg/ml rRNA solution was incubated for 15 minutes at 37°C in the presence of 40  $\mu$ gm of RNase A. 10  $\mu$ l of this rRNA per assay was used to assess RD-DP activity under conditions described in Methods. 100% activity was equivalent to 894 cpm/assay.
- 3 100  $\mu$ l of rRNA was treated with 40  $\mu$ gm of RNase A and 1.08  $\mu$ gm of RNase T<sub>1</sub> for 15 minutes at 37°C. Assays were carried out as described in 2.
- 4 DD-DP activity was assessed as described in Methods, using the same enzyme preparation as in 1.
- 5 Reaction was carried out for 1 hour. 100% activity is equivalent to 2,109 cpm/assay.
- 6 5  $\mu$ l of DNase I (1 mg/ml) and 10  $\mu$ l of 0.1M MgCl<sub>2</sub> were added to the assay after incubating for 30 minutes, followed by a further incubation of another 30 minutes.
- 7 10  $\mu$ gm RNase A was added after 30 minutes of incubation, followed by a further 30 minute incubation.
- 8 100% activity is equivalent to 670 cpm/assay.
- 9 150  $\mu$ gm of rRNA was incubated for 30 minutes at 37°C after which it was heated for 15 minutes at 70°C.
- 10 150  $\mu$ gm of rRNA was treated with 0.1  $\mu$ gm DNase I in the presence of 5 mM MgCl<sub>2</sub> for 30 minutes at 37°C, after which it was heated at 70°C for 15 minutes to destroy the DNase I. The rRNA-dependent activity using the template heated in the absence of DNase (see 9 above) was taken as 100%.



On DNase I treating the rRNA, and then inactivating the DNase by heating at 70°C it has been observed that only 39.4% of the activity of a control experiment in which RNA alone was heated, could be recovered. This may be due to several reasons: first, the RNA may be contaminated with DNA so that some of the activity detected may be DNA-directed; secondly, the DNase I solution may have been contaminated with RNase so that some of the template activity could have been destroyed by this nuclease; and, thirdly, some of the DNase I activity may have remained after the heat inactivation and consequently destroyed part of the product. Heating of the template itself has lowered its efficiency in directing DNA synthesis. For example, only 72.2% of the activity observed with the nonheated rRNA remained after heating the template at 70°C. This may be due to the removal of certain primers necessary to initiate activity.

(6) *TEMPLATE SPECIFICITY:*

(a) *Initial experiments -*

The (high MW) RD-DP obtained upon fractionation of an RNase treated extract was used for a preliminary study on the efficiency of certain RNA templates (Table VII). It was found that the three naturally occurring polyribonucleotides examined were utilized by the enzyme, although to different extents. *E. coli* rRNA resulted in the greatest activity, although QSRNA, which was present at a lower concentration, might prove to be most efficient on a weight basis. The use of yRNA for such studies could be criticized on the grounds that it is not highly purified, and so a further experiment was carried out in which the yRNA was first subjected to RNase treatment and then assayed for its template activity. This treatment completely destroyed its template activity,

TABLE VII

RNA-DEPENDENT DNA POLYMERASE ACTIVITY OF RAT THYMUS IN THE  
PRESENCE OF VARIOUS RNA TEMPLATES

<u>Template</u>	<u>Total Activity</u>		<u>-Endogenous Activity</u>	
	<u>(pmoles)</u>	<u>(%)</u>	<u>(pmoles)</u>	<u>(%)</u>
-RNA	0.0432	(32.3)	-	-
16S+23S rRNA	0.1338	(100)	0.0906	(100)
Q8RNA	0.0904	(67.5)	0.0472	(52.0)
yRNA	0.0667	(49.8)	0.0235	(25.9)
RNase treated yRNA	0.0003	(0.2)	-	-

1 ml of thymus extract was treated with 200  $\mu$ g of pancreatic RNase A for 30 minutes at room temperature, and subsequently fractionated on a Sephadex G 150 column. The endogenous and RD-DP activities were assayed as described in the legend to Fig. 1, and the most active fractions pooled and used as the source of enzyme. The RD-DP activity was then assayed in the absence and presence of yRNA, 16S+23S rRNA (each at a concentration of 5  $\mu$ g/assay) and Q8 RNA (at a concentration of 2.5  $\mu$ g/assay). In addition 100  $\mu$ g of yRNA was treated with 20  $\mu$ g of pancreatic RNase A for 30 minutes at room temperature and then used at a concentration of 5  $\mu$ g per assay to assess the RD-DP activity. 1 pmole is equivalent to 7,250 cpm. All assays were carried out in duplicate.

and furthermore decreased the endogenous activity from 32.3% (as compared to 16S+23S rRNA-dependent activity) to a mere 0.2%. These results with the RNase-treated yRNA support the conclusion that the yRNA itself is acting as the template and not some contaminating DNA.

(b)  $K_m$  for rRNA template -

Fig. 18 shows the effect of rRNA concentration on the velocity of the RDNA activity. As can be seen from the top figure, the rRNA saturation curve appears to be hyperbolic. The Lineweaver-Burk plot of this same data (lower figure) yields a straight line intercepting the ordinate, to give a  $K_m$  of 11.7  $\mu\text{gm}$  of rRNA per assay (i.e. 11.7  $\mu\text{gm}/50 \mu\text{l}$ ). The non-linear concentration curve for this enzyme (Fig. 17), however, prevents the determination of accurate kinetic parameters.

(c)  $K_m$  for yRNA template -

The  $K_m$  values for various RNA templates might be the key in indicating a preference on the part of the enzyme for certain types of RNA; for example, the lower the  $K_m$  value, the higher the affinity of the enzyme for that particular RNA.

The results of this study for yRNA are depicted in Fig. 19, and indicate a substrate inhibition effect. It is impossible to calculate a  $K_m$  for this template from the Lineweaver-Burk plot (Fig. 19 B).

(7)  $K_m$  for dTTP

The relationship between dTTP concentration and the velocity of the reaction is hyperbolic (Fig. 20, top). The double-reciprocal plot

FIG. 18. EFFECT OF rRNA TEMPLATE CONCENTRATION ON THE RD-DP ACTIVITY OF  
RAT THYMUS, AND ITS  $K_m$  AS DETERMINED BY A LINEWEAVER-BURK PLOT

The low MW RD-DP enzyme was used as the source of activity.  
Assay conditions were identical to those described in Methods except  
"cold" dTTP was deleted and the concentration of rRNA varied. Specific  
activity equals 7,250 cpm per pmole of  $^3\text{H}$ -TMP incorporated. All points  
on the graphs represent the average of duplicate assays.

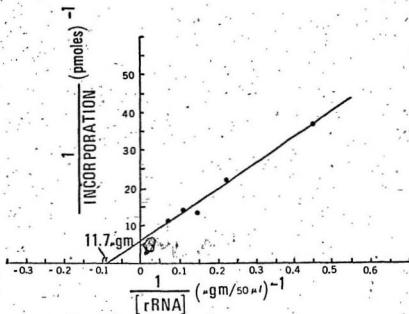
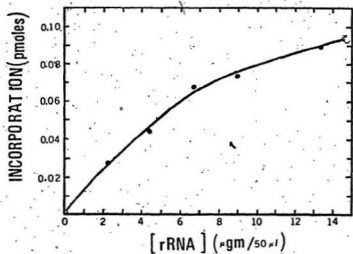


FIG. 18

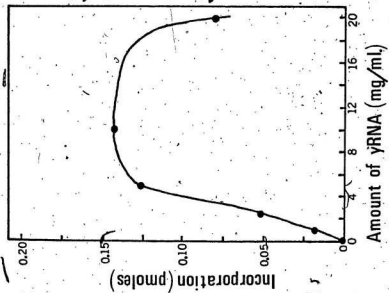
## FIG. 19. EFFECT OF VARIOUS CONCENTRATIONS OF yRNA ON THE RD-DP ACTIVITY

The low MW RD-DP was used as the source of activity. The activity was assessed as described in Methods except that yRNA was used as the template and its concentration was varied. Reactions were carried out for 30 minutes at 37°C and the radioactivity incorporated determined. The specific activity was 3,625 cpm/pmole of TMP incorporated.

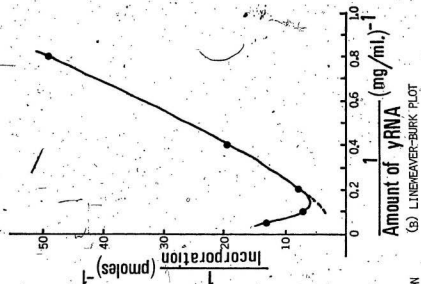
a. Incorporation vs yRNA concentration

b. Lineweaver-Burk plot

FIG. 19



(A). INCORPORATION VS yRNA CONCENTRATION



(B). LINEWEAVER-BURK PLOT

of the data (Fig. 20, bottom) allows a  $K_m$  of  $8.06 \mu M$  to be calculated for the substrate, dTTP.

#### (8) DIVALENT METAL REQUIREMENTS

Both RD-DP and DD-DP activities were dependent on the presence of a divalent cation (Fig. 21). In the case of the RD-DP,  $Mn^{++}$  was found to be more than twice as efficient as  $Mg^{++}$ , while the reverse was true for the DD-DP. The optimum for the DD-DP using  $Mg^{++}$  occurred between 1 and 2 mM  $Mg^{++}$ , while the RD-DP optimum was more broad and occurred at a higher  $Mg^{++}$  concentration (3-5 mM).  $Ca^{++}$  was found not to be used by the enzyme.

#### (9) EFFECT OF DITHIOTHREITOL ON THE RD-DP AND DD-DP ACTIVITIES

Fig. 22 shows the effect of dithiothreitol (DTT) on the DNA polymerases from rat thymus. With an incubation time of 15 minutes (top left hand figure), DTT had only small effects on either DD-DP or RD-DP. For an incubation time of 30 minutes, however, (top right hand figure) the DD-DP was stimulated as much as 35% by the presence of 4 mM DTT whereas the RD-DP was inhibited about 25% by the same DTT concentration.

The effect of dithiothreitol on the time-course of the reaction (Fig. 22, bottom) confirms that the DD-DP is stimulated by its presence, especially at incubation times exceeding 10 minutes, while the RD-DP is inhibited at longer incubation times. Possible explanations for this inhibition might be that DTT stabilizes an inhibitor of the enzyme or nucleases responsible for the breakdown of the template or the product. Alternately the RD-DP enzyme may contain disulfide bridges essential for maintaining an active conformation, and DTT may reduce such bridges and hence destroy the enzyme's activity, as has been reported for its inhibitory effect on RNase A (111).



FIG. 20. EFFECT OF dTTP CONCENTRATION ON THE RD-DP ACTIVITY OF RAT  
THYMUS

The RD-DP activity eluting in the low MW region of a Sephadex G 200 column was used as the source of enzyme. The reaction conditions were identical to those described in Methods, except the concentration of "cold" dTTP was varied. All points are the average of duplicate assays. The specific activity varied, depending on the concentration of "cold" dTTP.

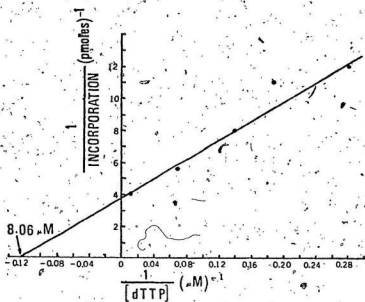
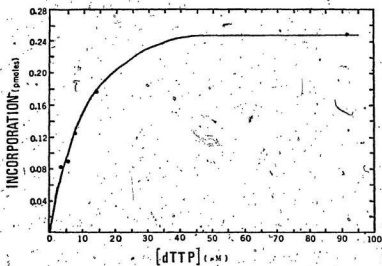


FIG. 20

FIG. 21. EFFECT OF DIVALENT CATIONS ON THE RD-DP AND DD-DP ACTIVITIES  
OF RAT THYMUS

RD-DP assays were carried out as described in Methods in the presence of various concentrations of either  $Mg^{++}$  or of  $Mn^{++}$  as indicated in the figure. The low MW RD-DP enzyme preparation was used as the source of activity. Reactions were carried out in duplicate and for 30 minutes at  $37^{\circ}C$ . The specific activity was equal to 3,625 cpm/pmole of TMP incorporated.

DD-DP activity was assessed as described in Methods, except that various concentrations of  $Mg^{++}$  or  $Mn^{++}$  were used. Incubation was also for 30 minutes at  $37^{\circ}C$ , and the reactions were carried out in duplicate. The specific activity was equal to 6,000 cpm/pmole of TMP incorporated.

A background of 60 cpm was subtracted from both activities.

FIG. 21

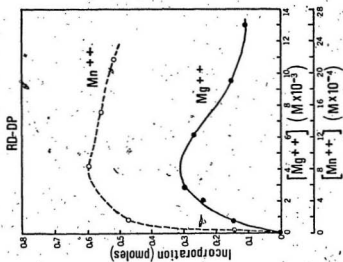
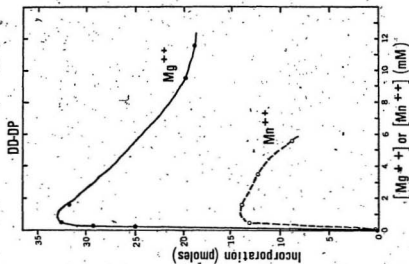


FIG. 22. EFFECT OF DITHIOTHREITOL ON THE RD-DP AND DD-DP ACTIVITIES  
FROM RAT THYMUS

Rat thymus extract was prepared and fractionated on a Sephadex G 200 column as usual (see top of Fig. 14), except for the absence of DTT in Buffer A. Reaction conditions were as described in Methods, except that the concentration of DTT was varied.

Upper diagrams - Effect of various concentrations of DTT on the two activities for 15 and 30 minute incubation times.

100% activity for the RD-DP is equivalent to 165 cpm (after subtracting background) for 15 minutes, and 295 cpm for 30 minutes. 100% activity for the DD-DP is equal to 91,891 cpm for 15 minutes, and 140,142 for the 30 minute assay.

Lower diagram - Time course of the two activities in the absence of and presence of 2 mM and 4 mM DTT.

The specific activity for the RD-DP reaction was 3,625 cpm/pmole of TMP incorporated, and that for the DD-DP reaction 6,000 cpm/pmole.

All points in the figure represent the average of duplicates.

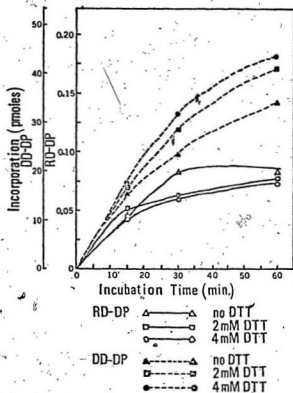
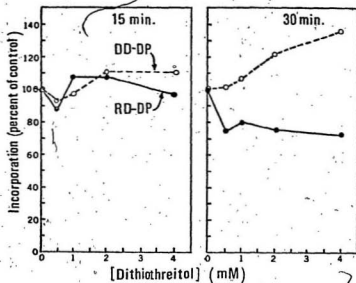


FIG. 22

(10) EFFECT OF MONOVALENT IONS ON THE DNA POLYMERASE ACTIVITIES

Fig. 23 shows the effect of KCl on the RD-DP and DD-DP activities. In the case of the DD-DP, the presence of KCl appears to inhibit the activity whereas the RD-DP activity is first stimulated and almost doubles in activity at a concentration of approximately 40 mM KCl, after which the activity decreases. It is not known whether these are specific effects due to KCl or non-specific ionic strength effects. Furthermore, I wish to point out that the ionic strength in the two assays is not identical. The initial ionic strength in the DD-DP assay (due mainly to tris-maleate buffer), is higher than that in the RD-DP assay (which contains a lower concentration of tris-HCl buffer). Stimulation of the DD-DP activity might thus occur at lower ionic strengths than were used in this experiment.

(11) EFFECT OF BOVINE SERUM ALBUMIN ON THE DNA POLYMERASE ACTIVITIES

Fig. 24 shows the effect of various concentrations of BSA on the RD-DP and DD-DP activities. The presence of 0.5 mg/ml of BSA resulted in a stimulation of both activities. The DD-DP was stimulated approximately 20% and the RD-DP approximately 25%. Higher concentrations appeared to inhibit the RD-DP to a small extent.

(12) EFFECT OF pH ON THE RD-DP AND DD-DP ACTIVITIES

The RD-DP appears to be most active in the relatively narrow pH range of approximately 8.5 - 9.0 (Fig. 25). The enzyme further appears to lose almost all activity with only a slight increase in pH above 9. The DD-DP on the other hand has a broader pH optimum ranging from pH 7 to 8. The higher activity at pH 9 for the RD-DP may not be due to an effect of pH on the enzyme itself, but to an effect on the template since polynucleotides at a basic pH exist in a more extended form, improving their efficiency as templates.

FIG. 23. EFFECT OF SALT CONCENTRATION ON THE RD-DP and DD-DP ACTIVITIES  
FROM RAT THYMUS

Buffer A was modified by omitting the KCl and a rat thymus extract was prepared and fractionated as usual (see Fig. 14 top) on a Sephadex G 200 column equilibrated with the same buffer. Reaction conditions were as described in Methods except that water was replaced by various concentrations of KCl. The specific activity of  $^3\text{H}$ -TTP for the RD-DP assay was 3,625 cpm/pmole and for the DD-DP, 6,000 cpm/pmole. All points are the average of duplicate assays.



FIG. 23

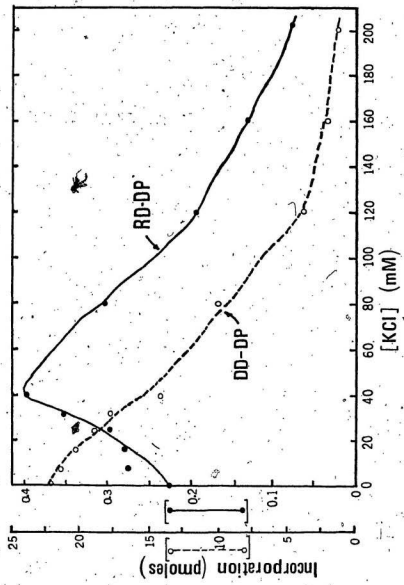


FIG. 24. STABILIZING EFFECT OF BOVINE SERUM ALBUMIN ON THE RD-DP AND  
DD-DP ACTIVITIES FROM RAT THYMUS.

Assays were carried out as described in Methods except that the water was replaced by various quantities of bovine serum albumin (BSA). All reactions were carried out in duplicate and the background subtracted. The specific activities are 3,625 cpm and 6,000 cpm per pmole of TMP incorporated for the RD-DP and DD-DP respectively.

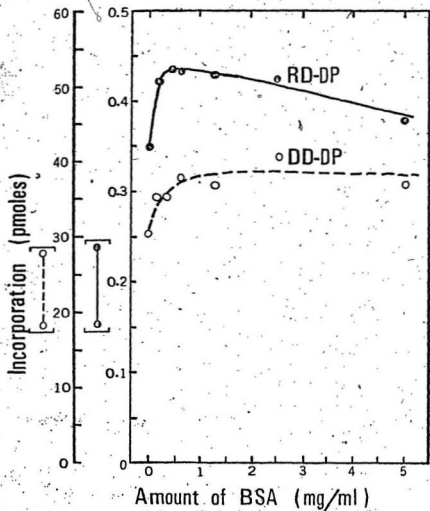


FIG. 24

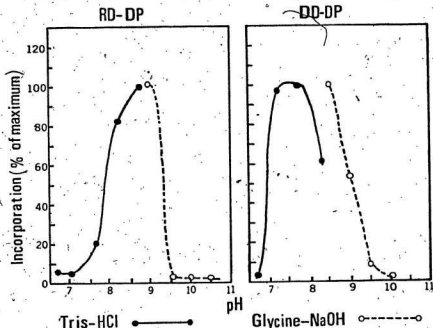


FIG. 25. EFFECT OF pH ON THE DNA POLYMERASES FROM RAT THYMUS

For experiments in the pH range of 6.5 to 8.5 1 ml aliquots of enzyme in Buffer A were titrated with either tris-OH, or HCl to give the appropriate pH, and the volume of each was adjusted with a tris-HCl buffer of the same pH to give identical volumes for each aliquot. Assays were then carried out for either 15 minutes (DD-DP) or 30 minutes (RD-DP) at 37°C as described in Methods except that the pH of each buffer mix in the assays was adjusted to the appropriate pH. 100% activity for the RD-DP and DD-DP is equivalent to 462, and 64,265 cpm respectively.

For studies conducted in the pH range of 8.5 to 10.5, the thymus homogenate was prepared in Buffer B. The pH of 1 ml aliquots was adjusted by the addition of either 1 M glycine or 2 M NaOH. Assay conditions were modified appropriately as above except that the buffer system was glycine-NaOH. In the case of the DD-DP the reaction was carried out for 15 minutes, and for the RD-DP for 30 minutes at 37°C. 100% activity for the RD-DP and DD-DP activities was equal to 1,111 and 102,304 cpm respectively. The specific activity was 3,625 cpm/pmole of  $^3\text{H}$ -TMP incorporated for the RD-DP, and 6,000 cpm/pmole for the DD-DP.

All reactions were carried out in duplicate and the average plotted.

(13) EFFECT OF TEMPERATURE ON THE RD-DP AND DD-DP ACTIVITIES:

(a) Temperature optimum -

Fig. 26 shows the effect of temperature on both the RD-DP and DD-DP activities. It is evident from the data that the optimum for the RD-DP is very narrow and lies approximately between 34 and 37°C. Temperatures slightly above 37°C result in a rapid loss of activity. DD-DP on the other hand, has a broad temperature optimum between 34 and 44°C. The activities of the two enzymes at 44°C are remarkably different: the RD-DP retains only about  $\frac{1}{10}$  of the activity observed at 37°C, whereas the DD-DP activity is almost in the optimum range.

(b) Heat inactivation studies of the high molecular weight enzymes -

In general, different enzymes might be expected to display different rates of inactivation on being exposed to heat. This property was examined in an attempt to establish whether the partially purified RD-DP and DD-DP activities from rat thymus are catalysed by the same or different enzymes (Fig. 27). The DD-DP activity decreases in a logarithmic fashion with a half-time ( $t_{1/2}$ ) of 22 minutes, and its rate of inactivation is less than that of the endogenous and RD-DP activities. Inactivation of the RD-DP and endogenous activities appears to be biphasic, the initial inactivation rates being quite rapid, with a half-time of 2-3 minutes. The second phase of inactivation on the other hand was slower for both activities, the endogenous having a  $t_{1/2}$  of 10 minutes, and the RD-DP a  $t_{1/2}$  of 18.5 minutes. However, upon correcting the  $t_{1/2}$  for the RD-DP (corrected  $t_{1/2}$  = 24 minutes) it was found to approach that of the DD-DP (Fig. 27B). Although the RD-DP (presumably enzyme not complexed with an endogenous RNA template) was expected to be less stable than the endogenous activity, this did not prove to be so.

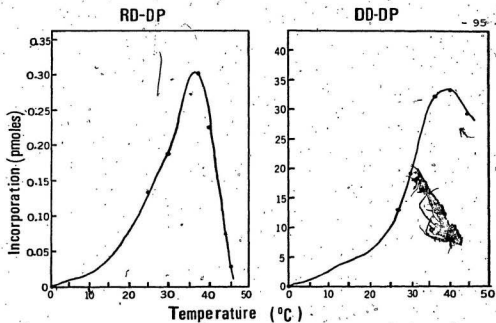


FIG. 26. EFFECT OF TEMPERATURE ON THE DNA POLYMERASES FROM RAT THYMUS

Assay conditions were as described in Methods. Reactions were carried out in duplicate for 30 minutes at different temperatures. The specific activity for the RD-DP activity was 3,625 cpm and for the DD-DP 6,000 cpm per pmoles of TMP incorporated.

FIG. 27. HEAT INACTIVATION OF THE RD-DP, ENDOGENOUS, AND DD-DP  
ACTIVITIES FROM RAT THYMUS AT 44°C

The enzyme preparation was obtained as described in the legend to Table VII. 25  $\mu$ l aliquots were incubated separately for various lengths of time at 44°C after which they were kept ice-cold until ready to be assayed for the RD-DP, the endogenous, and DD-DP activities. The three activities were then determined as described in the legends to Fig. 1 (endogenous and RD-DP) and Methods (DD-DP).

A: 100% activity for the RD-DP, endogenous, and DD-DP activities is equivalent to 1,058, 479, and 160,318 cpm respectively.

B: The half-time for the RD-DP activity was corrected by subtracting the amount of activity due to the endogenous template. 100% activity is equal to 579 cpm.

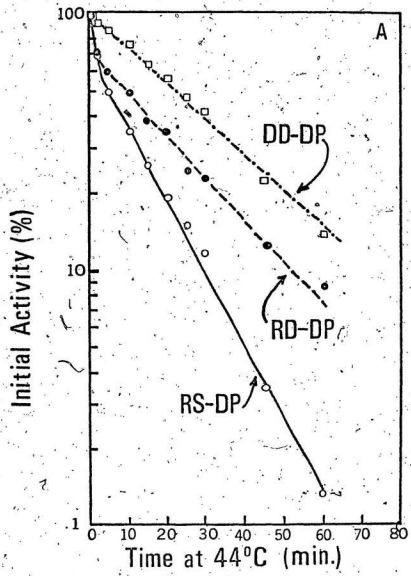


FIG. 27' (A)



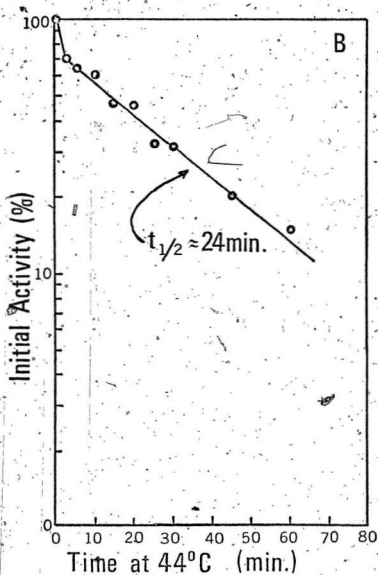


FIG. 27 (B)

(D) DETECTION AND PRELIMINARY CHARACTERIZATION OF A LOW MW DD-DP:

(1) ACTIVITY FROM RAT LIVER NUCLEI -

The RD-DP activity present in rat liver nuclei had a higher specific activity, and was less contaminated with DD-DP, than the enzyme preparation from thymus. It was therefore of interest to examine whether isolated nuclei could serve as a starting material for the preparation of a low MW RD-DP activity free of DD-DP activity. This proved to be unsuccessful. The profile of the activities from a RNase A treated supernatant of a nuclear extract that had been treated with 0.23% (w/v) DOC (Fig. 28) showed very little RD-DP activity at the low MW position of the column, although a low MW DD-DP peak was apparent. The lack of a low MW RD-DP peak may be due to one or more reasons. The enzyme may have lost activity due to the higher purity, or the RNase A may not have been active in the presence of DOC. This is a problem that remains to be pursued.

(2) ACTIVITY FROM RAT THYMUS

The observation of a distinctly separate low MW DD-DP activity in rat liver nuclei raised the question as to whether this was also the case with rat thymus. It may be possible that "tailing" of the high MW DD-DP enzyme may have hidden such an activity. Hence several approaches have been taken in determining whether this was the case.

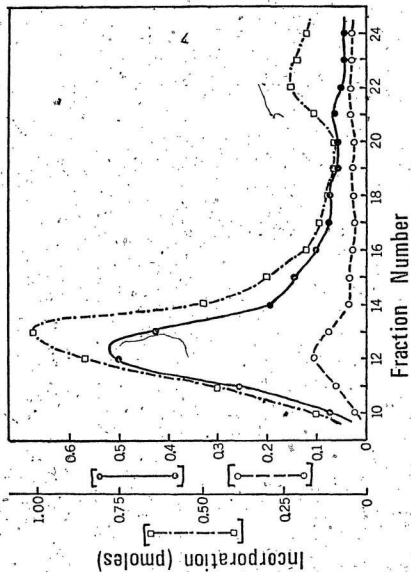
The first approach consisted of doing a heat inactivation study of the activities eluting in the region of the low MW RD-DP. A biphasic inactivation curve for the DD-DP would suggest the possible presence of two different DD-DP enzymes. Fig. 29 shows that this was the case. In addition to a rapidly inactivated DNA-dependent activity with an apparent  $t_{1/2}$  of 4 minutes (corrected  $t_{1/2} = 2$  minutes), there appeared to be an activity with a half-time of 23 minutes. 77% of the total DD-DP activity

FIG. 28. SEPHADEX G 200 ELUTION PROFILE OF RAT LIVER NUCLEAR DNA

POLYMERASES

Liver nuclei were prepared as described in Methods and 1 ml of frozen and thawed nuclei were treated with 50  $\mu$ l of a 5% DOC solution for 15 minutes at 0-4°C. This was followed by centrifugation at 7,000 g for 20 minutes. The supernatant was then treated with 400  $\mu$ gm of RNase A for 3 hours at room temperature, followed by fractionation on a Sephadex G 200 column. The fractions were then assayed for the RD-DP (●—●), endogenous DNA polymerase (○—○), and DD-DP (□---□) activities as described in Methods. Specific activities were as follows: 3,625 cpm/pmole of TMP incorporated for the RD-DP and endogenous DNA polymerase, and 6,000 cpm/pmole for the DD-DP.

FIG. 28



in this region was of the rapidly inactivated form whereas the remaining 23% was of the more stable form. The more stable enzyme may be due to "tailing" of the high MW enzyme whose  $t_{1/2}$  has been shown to be 22 minutes (refer to Fig. 27), almost identical to the more stable DNA dependent activity eluting in the low MW region.

In the same experiment, the stability of the low MW RD-DP activity was examined, and like the high MW RD-DP activity, it proved to be biphasic. However, a much larger proportion (92.4%) of rapidly inactivated activity (corrected  $t_{1/2}$  = 1.5 minutes) as compared to the more stable activity ( $t_{1/2}$  = 23 minutes) was present. The half-times of the more stable activities for both RNA- and DNA- dependent DNA polymerases are identical suggesting that these activities are due to the same enzyme. Part of the RD-DP activity (7.6%) may be due to contaminating DNA in the RNA template preparation, or due to use of RNA as a template by the contaminating high MW DD-DP. The corrected half-times of the rapidly inactivated activities are too close to say at this moment whether they are due to the same or different enzymes.

The second approach for detecting a low MW DD-DP in rat thymus involved the use of the inhibitor N-ethylmaleimide (NEM), which was reported by Smith and Gallo (119) to inhibit completely the high MW DD-DP (polymerase I, 150,000 - 160,000 MW) from normal human lymphocytes, while the low MW DD-DP (polymerase II, 30,000 MW) remained unaffected. The rationale for this approach was that the selective inhibition of the DD-DP eluting in the high MW complex might confirm the presence of a second DD-DP of a lower MW. Hence the effect of NEM was examined and the results (Table VIII) indicate that a DNA-dependent activity relatively resistant to NEM is indeed

FIG. 29. HEAT INACTIVATION OF THE LOW MW RD-DP AND DD-DP FROM RAT THYMUS

A low MW RD-DP enzyme preparation from rat thymus was heated for various lengths of time at 44°C, and both RD-DP activity and DD-DP activity present in the preparation were assessed as described in Methods. 100% activity for the RD-DP is equivalent to 698 cpm, whereas for the DD-DP it is equivalent to 9,653 cpm. The specific activities were as described in Fig. 28. All points represent the average of duplicate assays.

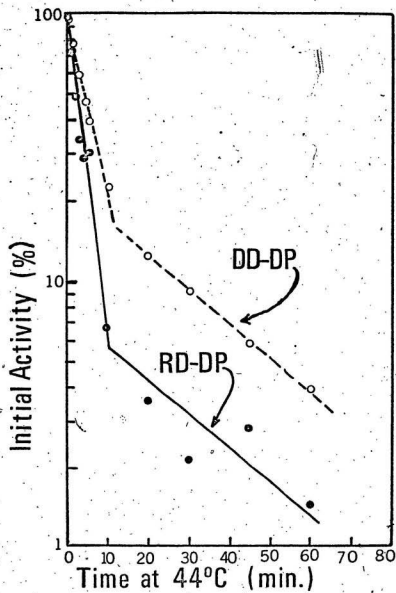


FIG. 29 (A)

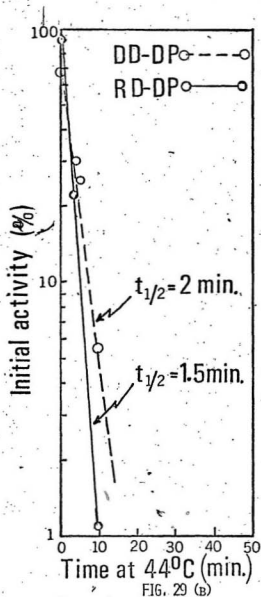


FIG. 29 (b)

Half-times for the rapidly inactivated first components were corrected graphically as described by Menzies et al. (118) from a plot of the difference between the observed percent activity and the percent activity of the second slowly inactivating component.



present in the low MW RD-DP preparation whereas the high MW DD-DP is almost completely sensitive to the inhibitor. This finding is further supported by Fig. 30 which shows the profile of the DNA polymerases from a rat thymus extract that had been extensively treated with RNase A and subsequently with NEM prior to fractionation. The inhibitor appears to have reduced the "tailing" of the high MW DD-DP, making apparent a second DD-DP peak of lower MW. The position of this second peak appears to coincide with that of the RD-DP activity, suggesting that the two activities may be due to the same enzyme. It was interesting to note in a control experiment in which the thymus extract was treated with only NEM (results not shown) that some of the RD-DP activity was shifted to a continuous series of different MWs on the Sephadex G-200 column, perhaps indicating that the binding of the enzyme to the endogenous template is in some way influenced by sulfhydryl groups.

(E) EFFECT OF VARIOUS INHIBITORS ON THE RD-DP AND DD-DP ACTIVITIES:

(1) EFFECT OF RIFAMYCIN DERIVATIVES -

Because of the current interest in the use of specific inhibitors of RD-DP for cancer chemotherapy, the next experiments were directed towards investigating the effect of some of these inhibitors on the RD-DP from untransformed rat thymus cells. Furthermore an attempt was made to establish a difference between the DD-DP and the RD-DP, hence the effect of the inhibitors on the DD-DP was also investigated.

For these studies it was necessary to dissolve the inhibitors in dimethylsulfoxide (DMSO). It was observed, however that this solvent greatly stimulated the enzyme activities [perhaps due to its effect on the templates as a result of its high dielectric constant (120)]. It was therefore

TABLE VIII.

EFFECT OF N-ETHYLMALEIMIDE ON THE HIGH MW DD-DP AND THE DD-DP  
CO-FRACTIONATING WITH THE LOW MW RD-DP

	<u>-NEM</u>	<u>+NEM</u>
DD-DP from peak I <sup>1</sup>	100%	13.62%
DD-DP from peak II <sup>2</sup>	100%	76.23%

High and low MW enzymes were prepared and assays carried out in duplicate, as described in Methods, in the presence and absence of 200  $\mu$ gm of NEM per ml. Reactions were for 30 minutes at 37°C and the specific activity was 6,000<sup>1</sup> cpm per pmole of TMP incorporated.

<sup>1</sup>100% activity is equal to 15,363 cpm.

<sup>2</sup>100% activity is equal to 4,383 cpm. Peak II refers to the DNA-dependent activity eluting with the low MW RD-DP.

FIG. 30. ELUTION PROFILE OF THE DNA POLYMERASES FROM RAT THYMUS AFTER  
RNase A AND N-ETHYLMALIMIDE TREATMENT

1 ml of a 25% (w/v) rat thymus homogenate in Buffer A was treated with 400  $\mu$ g of RNase A for 8 hours at room temperature. 200  $\mu$ g N-ethylmaleimide was then added to the treated extract, and subsequently fractionated through a Sephadex G200 column. The activities were assessed as described in Methods, and reactions were carried out for 30 minutes at 37°C. The specific activities were 3,625 cpm/pmole for the endogenous and RD-DP activities, and 6,000 cpm/pmole for the DD-DP.

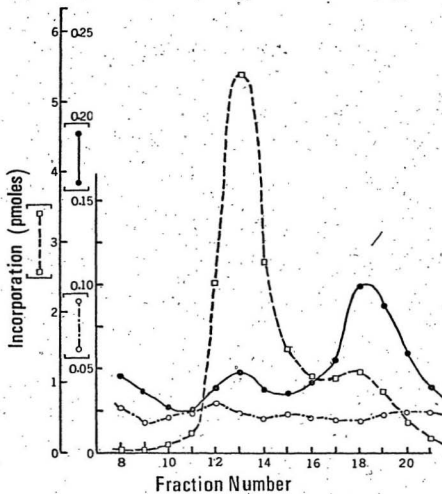


FIG. 30

necessary to examine the time dependence of the two activities under these conditions (Fig. 31). The RD-DP was found to be linear for at least 30 minutes, whereas the DD-DP activity was linear for only 15 minutes.

The effect of various inhibitors are shown in Fig. 32, and Table IX summarizes these results. The effects of the various rifamycin derivatives (M/14, rifampicin, AF/AP, rifamycin SV, AF/DNF 1, AF/ABDP-cis, and AF/013) on the RD-DP of rat thymus were found to be quite similar to their effects on the viral enzyme as reported by Smith et al. (47), Ting et al. (46), and Yang et al. (48), and could in fact be classified in the same manner as they have been classified for the viral enzyme.

Some notable differences were observed in the effects of inhibitors on the RD-DP and DD-DP activities. For example, AF/AP at a concentration of 100  $\mu\text{g}/\text{ml}$  was found to inhibit the RD-DP about 25% whereas the DD-DP was not affected, and AF/DNF I at the same concentration inhibited about 95% of the RD-DP whereas only 21% of the DD-DP activity was lost. AF/ABDP-cis at a concentration of 200  $\mu\text{g}/\text{ml}$  eliminated 96% of the RD-DP activity and 59% of the DD-DP. On the other hand, rifampicin, M/14, AF/013, and rifamycin SV had little differential effect on the two activities.

In addition to the rifamycin derivatives, the effects of other inhibitors (such as actinomycin D, ethidium bromide, N-ethylmaleimide, and p-chloromercuribenzoate) were also examined.

## (2). EFFECT OF ACTINOMYCIN D AND ETHIDIUM BROMIDE ON THE RD-DP AND DD-DP ACTIVITIES

Actinomycin D is known (121) to require double-stranded regions containing G-C complementary bases in order to intercalate and block the use of the template by the enzyme. This inhibitor was found to inhibit the RD-DP activity slightly more than the DD-DP (92% compared to 80% for

the two activities respectively, at a concentration of 100  $\mu\text{g}/\text{ml}$ ) (Fig. 32 and Table IX). These findings could possibly indicate that about 90% of the activity observed in the presence of RNA is DNA-dependent. It is possible, however, that the effect of actinomycin D is due to inhibition of a second stage of the RNA-dependent reaction, that is the antibiotic may affect the use of newly synthesized DNA for the synthesis of a complementary DNA, as has been observed in the case of the viral enzyme (62). Hence, the time course of the reaction was determined in the presence and absence of actinomycin D, and the results (Fig. 33) indicate that possibly two reactions may be involved, one that is sensitive to actinomycin D, and one that is not. The insensitive one appears to occur at a much slower rate than the sensitive one.

Ethidium bromide is believed to act in much the same way as actinomycin D. This inhibitor, however, will intercalate into both DNA and RNA (124, 125). Ethidium bromide was found to inhibit about 35% of the RD-DP, and about 39% of the DD-DP activity (Fig. 32 and Table IX).

### (3) EFFECT OF SULPHYDRYL REAGENTS ON THE RD-DP AND DD-DP ACTIVITIES

Perhaps the most interesting results of these inhibition studies are those obtained with the sulphydryl reagents N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB). It was found that NEM, at a concentration of 200  $\mu\text{g}/\text{ml}$ , inhibited over 99% of the DD-DP (after 10 minutes preincubation at 0°) and only 20% of the RD-DP activity. PCMB was found to inhibit the DD-DP about 70% and the RD-DP only 15% at a concentration of 20  $\mu\text{g}/\text{ml}$ . These results are consistent with the earlier finding that dithiothreitol in the reaction mixture is of no benefit to the RD-DP activity whereas its presence stabilizes the DD-DP. In this respect the properties

of the RD-DP from rat thymus differ from the reported properties (9, 10, 18, 24, 26) of the RD-DP of viral origin, which does require sulfhydryl reducing agents to maintain prolonged enzyme activity.

FIG. 31. KINETICS OF RD-DP and DD-DP ACTIVITIES FROM RAT THYMUS IN THE  
PRESENCE OF DIMETHYLSULFOXIDE (DMSO)

The low MW RD-DP and high MW DD-DP activities from a Sephadex  
G 200 column were used as the source of enzyme. Reaction conditions  
were identical to those described in Methods except that 5  $\mu$ l of water  
was replaced by 5  $\mu$ l of DMSO, and reactions were carried out for 30  
minutes at 37°C. Specific activities were identical to those in Fig. 30.



Fig. 32: EFFECT OF VARIOUS INHIBITORS ON THE RD-DP AND DD-DP ACTIVITIES  
FROM RAT THYMUS

Enzymes were prepared the same way as described for Fig. 31. The inhibitors rifamycin SV, AF/AP, AF/O13, AF/DNF I, AF/ABDP-cis, M/14, rifampicin, and ethidium bromide were dissolved in DMSO at appropriate concentrations and added to assay mixtures in place of 5  $\mu$ l of water. Actinomycin D, and NEM were prepared in Buffer A, and pCMB in 1 M NaOH, and neutralized with HCl until the pCMB just remained in solution (i.e. approximately pH 10.3).

Assays were carried out as described in Methods except that 5  $\mu$ l of water were replaced by 5  $\mu$ l of inhibitor solutions that were diluted with the same solvent they were dissolved in to give the appropriate final concentration denoted in the figures. The RD-DP reactions were incubated for 30 minutes at 37°C and those for the DD-DP for 15 minutes in the case of the inhibitors dissolved in DMSO, and for 30 minutes for all other inhibitors. 100% activity was taken as the activity observed in the presence of 5  $\mu$ l of the solvent used for that particular inhibitor. All assays were done in duplicate.

FIG. 32

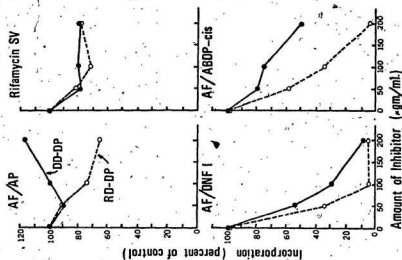


FIG. 31

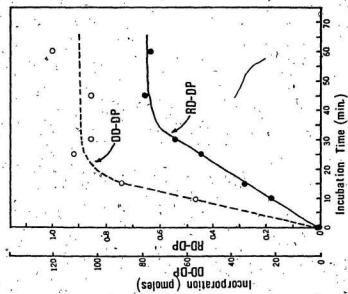


FIG. 32 (CONTD.)

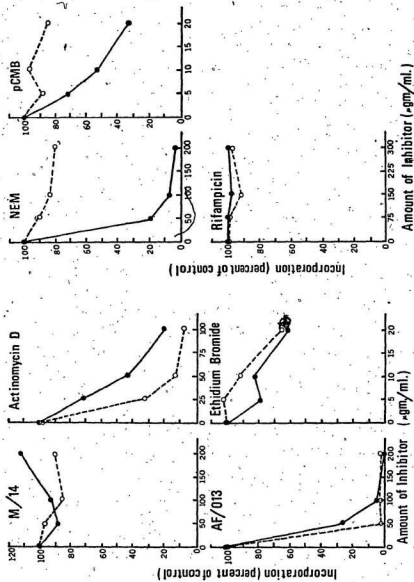


TABLE IX,

## EFFECT OF VARIOUS INHIBITORS ON THE DNA-POLYMERASES


## FROM RAT THYMUS

<u>Inhibitor</u>	<u>Inhibitor Concentration (<math>\mu</math>g/ml)</u>	<u>RD-DP (% inhibition)</u>	<u>DD-DP (% inhibition)</u>
I. Rifamycin derivatives:			
A. Ineffective inhibitors:			
M/14	(100)	15.8	9.8
Rifampicin	(150)	8.0	2.0
B. Moderately effective inhibitors:			
AF/AP	(100)	25.1	0
Rifamycin SV	(100)	28.5	19.7
C. Potent inhibitors:			
AF/DNF I	(100)	95.2	71.1
AF/ABDP-cis	(100)	65.5	24.5
	(200)	96.0	59.0
AF/O13	( 50)	97.4	72.7
	(100)	96.9	96.5
II. Sulfhydryl reagents:			
A. N-ethylmaleimide			
	(100)	16.0	93.0
	(200)	20.0	96.8
B. p-chloromercuribenzoic acid			
	( 10)	2.5	46.7
	( 20)	14.7	69.8
III. Other inhibitors:			
A. Actinomycin D			
	( 50)	87.5	56.4
	(100)	92.0	80.2
B. Ethidium bromide			
	( 10)	7.5	17.1
	( 20)	34.5	38.7

This is a summary of the results shown in Fig. 32, and experimental details described for that figure are applicable.

FIG. 33. EFFECT OF ACTINOMYCIN D ON THE RD-DP ACTIVITY

The low MW RD-DP enzyme was used for all assays. Reaction conditions were as described in Methods and were carried out for various lengths of time at 37°C in the absence (top curve) or in the presence (lower curve) of 25 µgm of actinomycin per assay. The specific activity was equal to 3,625 cpm/pmole of TMP incorporated. All assays were done in duplicate and the average plotted.



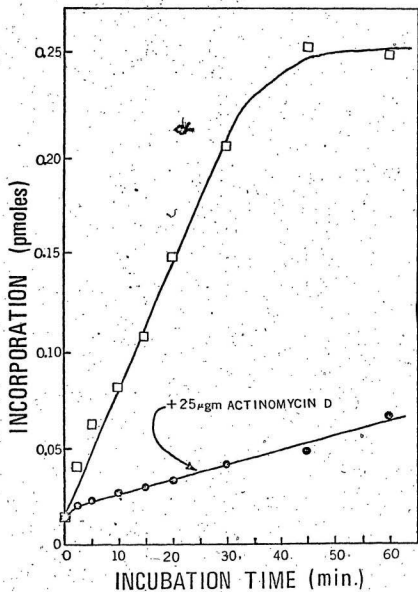


FIG. 33

## DISCUSSION

(A) EVIDENCE IN SUPPORT OF AN RNA-DEPENDENT DNA POLYMERASE IN  
MAMMALIAN CELLS

"Real" RNA-dependent DNA polymerases have been defined as those activities capable of synthesizing DNA from natural RNA templates (13). This definition does not preclude the possibility that the same enzyme might also be capable of using other templates (for example, synthetic RNAs or DNA). It is of interest to note that the "reverse transcriptase" (RD-DP) of cancer viruses transcribes DNA templates more efficiently than RNA templates (32, 36).

The criteria essential for defining RNA-dependence have been outlined in Section A, 3 - *Literature Review* (refer to p. 4). With regard to labelling the activity studied in this present work as an RNA-dependent DNA polymerase, only certain of these criteria have been met: the activity is susceptible to RNase, requires all four deoxyribonucleoside triphosphates for maximum activity (hence it is not a terminal transferase), and the product is sensitive to DNase and resistant to RNase. Hybridization experiments would provide absolute proof for the presence of such an activity in rat tissues. Such studies are being planned although they have not yet been carried out.

Sensitivity to RNase in the case of the endogenous activity does not prove conclusively that RNA is the template since it has recently been reported that RNA may provide a primer for the DD-DP activity (128, 129). RNase treatment may therefore be eliminating such primers and consequently reducing the activity. On the other hand, RNase treatment of the exogenous RNA template, resulting in the elimination of

a large part of the activity (Table VI), provides strong evidence that RNA is the template for DNA synthesis. It is unlikely that RNA in such a situation would act only as a primer, unless DNA is present in the RNA preparation (or in the enzyme preparation) which will serve as a template only in the presence of an RNA primer. Conversely a DNA primer for RD-DP might be postulated which would explain the partial elimination of activity upon DNase treatment (Table VI). It is more likely, however, that the partial sensitivity to DNase is actually due to the presence of some contaminating DNA in the RNA preparation which is used as a template by DNA-dependent DNA polymerase. For this reason, rRNA and Q $\beta$ RNA, which are of high purity, were used as templates. Surprisingly, it was the yeast RNA-dependent activity which was completely eliminated by RNase treatment (Table VII), clearly suggesting that RNA is the template and not some contaminating DNA. The incomplete inactivation of DNase by heat treatment is a possible reason for the reduced efficiency of DNase treated RNA, since any remaining DNase would destroy the newly synthesized product. Experiments in which the RNA preparation will be purified by centrifugation through a CsCl gradient after DNase treatment (83) are presently being planned.

Other arguments may be presented that are in favor of RNA acting as a template. The fact that a large fraction of the RD-DP activity can be separated from the high MW complex of the DD-DP after RNase treatment argues in favor of this activity being preferentially associated with RNA. Obviously, this activity is different from the high MW DD-DP since it uses an RNA template with a higher relative efficiency



than does the DD-DP. If a contaminating DNA were responsible for this activity, it is certainly not used very efficiently by the major DD-DP since the rRNA preparation elicited only 2.4% as much activity as the "activated" DNA with the high MW DNA polymerase (Fig. 5A). This indirectly argues against contaminating DNA being the template. The finding of a DD-DP activity in the RD-DP region, however, does suggest that either some of the DD-DP is also released by RNase treatment, or else that the RD-DP, once separated from its endogenous template, may use either RNA or DNA as a template. It is interesting that this second peak of DD-DP (refer to Fig. 30) accepts both "activated" DNA and native DNA with almost equal efficiency (data not shown).

The relative ratios of the DD-DP to RD-DP in the various rat tissues examined (see Table II) lends further support to the suggestion that RNA is the template. This is so because the ratios of the two activities after various times of storage vary greatly (although the same RNA preparation was used). The argument is that if the activity is due to a contaminating DNA then the relative ratio of DNA-/RNA-dependent activities should be constant, which it was not.

Partial resistance to actinomycin D also suggests that RNA is the template. That part of the reaction which is sensitive to the antibiotic may involve a second step of the reaction involved in the formation of double-stranded DNA.

#### (B) UNIFYING MODEL

The observation that prolonged RNase treatment can shift the RD-DP activity to a lower MW region is consistent with the suggestion that the activity is complexed to an endogenous RNA template. This

template may be partially or completely degraded during RNase treatment.

The possibility that the same enzyme or enzymes may direct both RNA and DNA-dependent activities must not be overlooked. In this case the efficiencies with which either RNA or DNA are used differ, depending on which enzyme is being studied. This suggestion is supported by the fact that in none of the experiments did RD-DP activity completely shift to the 120,000 MW region. The remaining activity in the void volume may represent the extent to which the DD-DP in that region makes use of RNA as a template (that is, with very low efficiency), and the RD-DP activity in the 120,000 MW region, the extent to which the low MW DNA polymerase makes use of RNA (much higher relative efficiency). Alternately the two activities may represent different enzymes that specifically use either RNA or DNA only. More extensive purifications, should yield an answer to this question.

The results with DNase treatment indicate that the effects of RNase in releasing RD-DP from the high MW nucleic acid-protein complex are specific, since DNase at either high or low levels did not mimic the effect of RNase. In fact, DNase shifted the position of the DD-DP slightly without affecting the position of the RD-DP (Fig. 7).

The results with the prolonged DNase treatment may be explained by precipitation as Mg-protein complexes of the proteins (including DD-DP and RD-DP) released from the DNA by DNase treatment. The lack of precipitation in the absence of DNase but the presence of  $Mg^{++}$  may be due to the fact that the enzymes are still associated with nucleic acids and kept in solution by them.

The absence of DTT in the homogenizing medium has resulted in only a partial recovery of activity relative to the amount of activity recovered when it was present. Its addition just prior to the assay, however, did not have any beneficial effects on the RD-DP activity. This suggests that sulfhydryl groups which are essential for activity are slowly oxidized and hence the slow loss of activity. It appears that DTT prevents this slow oxidation during long periods of time, but is relatively ineffective for short periods during which little oxidation occurs. These observations suggest that the sulfhydryl groups may be deeply hidden within the enzyme molecule so that they are not easily accessible for oxidation. The results obtained with NEM and pCMB which only slightly inhibit this activity (Table IX) are consistent with this suggestion.

#### (C) COMPARISON OF RD-DP ACTIVITIES FROM RAT THYMUS AND RNA ONCOGENIC VIRUSES

The RD-DP activities from cellular and viral sources have been shown to differ in many respects. In all cases the activity from viruses had to be made accessible by treatment with nonionic detergents such as Nonidet P<sub>40</sub> or Triton X 100. This was not found to be the case, however, with the activity from rat thymus. Upon exposing the thymus supernatant to Triton X 100 (in the concentration range of 0.001% - 0.8%, data not shown), there was no apparent increase in activity, although some inhibition did seem to occur. This suggests that the source of the enzyme from the thymus is not a virus-like particle, although it could still be argued that viral components may be present in the tissue which

have not been assembled into intact virions. Teitz (130) has reported an RNA-dependent activity from "normal" rat thymus tissue cultures, and has shown its source to be type C particles on the basis of the density at which the particles banded in a sucrose gradient, and by the large increase in RD-DP activity upon treatment with nonionic detergents. Such cell cultures, however, are far from being normal. In fact, histological studies revealed that these cells differed greatly in morphology (within 24-48 hours from the beginning of the culture) when compared to freshly prepared rat thymus cells (131).

The properties of the cellular RD-DP activity are similar only in some respects to the properties reported for the viral enzyme. For example, DNA polymerases from both sources require all four deoxyribonucleoside triphosphates for maximum activity. Both activities respond to the divalent cations,  $Mn^{++}$  and  $Mg^{++}$  and not to  $Ca^{++}$ . The RD-DP from AMV has been shown to prefer  $Mn^{++}$  to  $Mg^{++}$  in the case of enzyme preparations contaminated with RNase, while the opposite was true of RNase-free preparations (24). The RD-DP activity from non-malignant rat thymus also prefers  $Mn^{++}$  and in this respect is also similar to the AMV enzyme. Assays for RNase activity however, have not been conducted and it is not known whether this nuclease is contaminating the enzyme preparation.

The activity from RSV requires the presence of dithiothreitol or mercaptoethanol for maximum activity (9, 10, 18, 24), and is highly sensitive to sulphydryl reagents (26), whereas that from rat thymus did not require dithiothreitol, and was relatively insensitive to sulphydryl reagents. The difference in sensitivity to sulphydryl reagents is of

great interest especially since the other inhibitors studied had effects similar to those reported for the viral enzyme.

There also appear to be differences in the MWs of the enzymes from rat thymus and viruses (see p. 7). However, although a MW of 120,000 has been estimated for the RD-DP from rat thymus, this may not be its true MW; the enzyme may still be associated with nucleic acid species. In fact, there is support for this in the observation that upon storing the enzyme preparation (eluting in the 120,000 MW region) at  $-20^{\circ}\text{C}$ , a small amount of activation of endogenous activity can be observed, consistent with the suggestion that the enzyme may still exist as a nucleoprotein complex. Both the viral (18, 22, 24) and mammalian enzymes appear to consist of subunits on the basis of the observation that the activity is less than proportional to enzyme concentration below a critical concentration (Fig. 17), presumably due to dissociation into constituent subunits.

In addition, the activity described in this work, as is also true for the viral enzyme, has the capacity to use externally added natural RNAs as templates, and in this respect it differs from RD-DP activities (from non-malignant cells) described by other workers (77, 79, 80), which are incapable of using externally added natural RNA templates.

#### (D) POSSIBLE FUNCTIONS

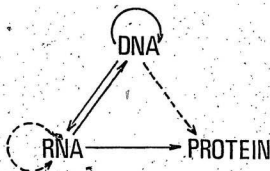
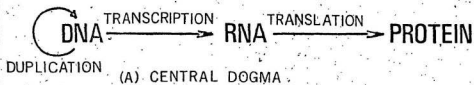
Although the Central Dogma of molecular biology proposed by Crick (7) in 1958 stated that "once information has passed into protein it cannot get out again", many molecular biologists felt that the transfer of information from RNA to DNA violated the Central Dogma. On the basis of the gross chemical composition of *E. coli* (1% DNA, 6% RNA, and

15% protein) (132), they intuitively visualized the information transfer process in cells as depicted in Scheme IV A [from Watson, p. 331 (133)]. Hence the initial indirect evidence (1, 2-5) for a reversal of the transcription process was not widely accepted. However, the discovery of an RNA-dependent DNA polymerase has raised serious questions in regard to the *Central Dogma*. This has led Crick (34) to clarify what the *Central Dogma* originally stated. In addition he has reclassified the types of information transfer that may or may not take place in biological systems as follows (134):

<u>General Transfers</u>	<u>Special Transfers</u>	<u>Unknown Transfers</u>
DNA → DNA	RNA → RNA	PROTEIN → RNA
DNA → RNA	DNA → PROTEIN	PROTEIN → DNA
RNA → PROTEIN	RNA → DNA	PROTEIN → PROTEIN

The General Transfers constitute modes of information transfer that occur in all cells. Special Transfers are those that do not occur in most cells, but may occur under special circumstances, and Unknown Transfers constitute those that never occur.

The more general distribution of RD-DP than originally thought during the early period following its discovery in RNA oncogenic viruses [as supported by reports in the literature (72-83, 85)], and this present work] suggests that the information transfer from RNA to DNA no longer constitutes a Special Transfer but rather a General Transfer. Hence a further revision of the *Central Dogma* is essential, as Crick suggested, if this mode of information transfer should prove to be widely distributed. We may now represent the *Central Dogma* as depicted in Scheme IVB. In this scheme, the solid arrows indicate General Transfers and the broken arrows Special Transfers. The Unknown Transfers remain as Crick



## SCHEME IV

CENTRAL DOGMA OF MOLECULAR BIOLOGY

suggested (134).

This revised version of the *Central Dogma* suggests that the function of RD-DP is to transfer information from RNA to DNA. The number of situations under which this information transfer may occur are numerous although largely speculative. It may occur during embryonic development, as <sup>R</sup>part of the process of differentiation (77, 94-97), in the specialization of immunocompetent cells for the production of specific antibodies (126), and even in the process of memory (70, 71). It is interesting that one of the theories of memory may suggest a role for RD-DP. For example, if information were to be coded into RNA, then this information could be stabilized by a reversal of the transcription process, yielding a more stable piece of information (DNA).

The role of an RD-DP in the amplification of rRNA genes in *Xenopus* oocytes has already been documented by a number of workers (86, 90) although their evidence is still questionable (91).

Arguments have been presented with regard to RNA having appeared before DNA in the evolutionary process (135, 136). If this were the case, the importance of RD-DP in the process of evolution would be evident.

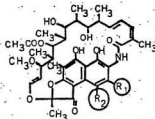
Thus it appears that the RD-DP in eukaryotic cells may prove to be very versatile in its biological role, although at this time any function assigned to this enzyme in such cells is purely speculative.



## APPENDIX I

### STRUCTURE OF RIFAMYCIN DERIVATIVES

This table has been modified from Smith et al. (57), and Ting et al. (56). The Pharmaceutical Code refers to the code used by Gruppo Lepetit Pharmaceutical Co., Milan, from whom the inhibitors were obtained. The compounds have been classified according to their effects on viral RD-DP (57). The Class A compounds have little or no effect on RD-DP, the Class B compounds have a moderate effect, and Class C are potent inhibitors.



PHARMACEUTICAL CODE	CHEMICAL NOMENCLATURE	R <sub>1</sub>	R <sub>2</sub>	CLASS
Rifampicin	3-[4-methylpiperazinyl-imino-methyl]rifamycin SV		-OH	A
N/14	Rifampicin B:N,N-diethylamide-sodium salt	-H		A
—	Rifamycin SV	-H	-OH	B
AF/AP	N-Demethylrifampicin		-OH	B
AF/O13	3-Formyl rifampicin SV:O-n-octyloxime	-CH=NO(CH <sub>2</sub> ) <sub>7</sub> CH <sub>3</sub>	-OH	C
AF/DNF1	3-(2,4-Dinitrophenylhydrazonemethyl)-rifamycin SV		-OH	C
AF/ABDP	2,6-Dimethyl-4-benzyl-4-demethyl-rifampicin		-OH	C

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PUBLICATIONS ARISING FROM THIS WORK

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